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TOXIN PRODUCTION AND IMMUNOASSAY DEVELOPMENT

I. PALYTOXIN

ANNUAL/FINAL REPORT

T.J.G. RAYBOULD

APRIL 18, 1991

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One hundred fifty mgs of purified lyngbyatoxin A were collected, isolated and delivered to USAMRIID. The specificity of the palytoxin CIEIA was investigated. Native palytoxin was the most reactive, N-acylated palytoxin and inactivated palytoxin were found to retain 10 to 100% serological reactivity. The marine toxins okadiac acid, tetrodotoxin, and lyngbyatoxin A showed no detectable reactivity in the immunoassay. Monoclonal antipalytoxin antibody 73D3 was found to be effective in neutralizing both in vitro and in vivo cytotoxicity of palytoxin. Synthesis of new palytoxin haptens with linkers at sites other than the terminal amino group were begun. Attempts to synthesize a palytoxin hapten linked at the hydroxal end of the molecule or in developing the oxidation/ reduction sequence needed for the tritiation of palytoxin have been unsuccessful. Possible corrective measures include the production of an anhydrous sample to minimize water interference.

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SUMMARY

The major accomplishments achieved during the course of this four year project are as follows:

- A method for the large scale extraction and purification of palytoxin from *Palythoa toxica* and *P. tuberculosa* was developed and optimized.
- Sufficient *Palythoa tuberculosa* was collected to enable the isolation of 650 mg of purified palytoxin. Of this amount, 308 mg was delivered to USAMRIID and the balance used at HBG for studies of palytoxin chemistry and immunology.
- Sufficient *Lyngbya majuscula* was collected to enable the isolation of 150 mg of purified lyngbyatoxin A.
- Five palytoxin-protein conjugates, linked via the amino terminus of palytoxin, wer prepared for use as immunogens and coating antigens for immunoassay development.
- Several approaches for the synthesis of alternative, non-amino linked, palytoxin conjugates were investigated. Selective functionalization of the primary hydroxyl group of palytoxin was not successful. Data indicates that palytoxin was successfully converted to palytoxin carboxylic acid, however, insufficient quantities of the acid were obtained for conclusive identification and conjugate synthesis.
- Several approaches to develop chemical methods for the tritium labeling of palytoxin were studied. No satisfactory answer to this problem was found.
- High titer, high avidity rabbit polyclonal antisera and high affinity mouse monoclonal antibodies against palytoxin were produced.
- Polyclonal and monoclonal antibodies (MAbs) have been purified with high yields and specific activities by the use of affinity chromatography on Protein A-Sepharose using discontinuous pH gradient elution.
- Sensitive and specific indirect competitive inhibition enzyme immunoassays (CIEIAs) have been developed using palytoxin-bovine serum albumin solid phase coating antigens with the above antibody preparations and alkaline phosphatase labelled-anti rabbit IgG and -anti mouse IgG conjugates. A sensitive indirect sandwich enzyme-linked immunosorbent assay (ELISA) has been developed using purified monoclonal antibody solid phase coating antigen with rabbit polyclonal antibody from rabbit 7 bleed 7 and alkaline phosphatase labelled-anti rabbit IgG conjugate.
- High activity alkaline phosphatase (AP) conjugates have been prepared with rabbit polyclonal immunoglobulins purified from rabbit 7 bleed 7, monoclonal antibody immunoglobulins purified from hybridoma 73D3 ascitic fluid, and palytoxin hapten (PTX-MCC). Two new direct CIEIA systems have been developed using the AP-73D3 MAb and AP-PTX-MCC conjugates on solid phases coated with BSA-PTX-MCC and 73D3 MAb respectively. These direct CIEIAs are as sensitive or more sensitive than the indirect CIEIA and involve one step less, with the result that they are more rapid, require fewer reagents, and are more cost effective.

- A sensitive direct sandwich ELISA has been developed using purified monoclonal antibody solid phase coating antigen with alkaline phosphatase labelled-rabbit 7 bleed 7 polyclonal antibody conjugate. This direct sandwich ELISA is as sensitive as the indirect sandwich ELISA and involves one step less, with the result that it is more rapid, requires fewer reagents, and is more cost effective.
- The PTX content of 70% ethanol extracts of *Palythoa tuberculosa* collected from Okala Island on 8/15/1987 was assayed on at least two occasions. On the first occasion, the indirect CIEIA and the indirect sandwich ELISA were employed. On the second occasion, the indirect CIEIA, the indirect sandwich ELISA, the two direct CIEIAs, and and the direct sandwich ELISA were all employed. There was good agreement between the palytoxin concentrations given by the the five EIA systems on the second occasion. There was also good agreement between these results and the PTX concentrations obtained on the first occasion using the indirect sandwich ELISA and indirect CIEIA. A "non-parallelism" phenomenon was observed in the results of the indirect sandwich ELISA and indirect CIEIA from the first occasion. A similar phenomenon was observed with the results of certain extracts on the second occasion in the indirect sandwich ELISA, but not in the indirect CIEIA or either of the direct CIEIAs.
- The direct CIEIA using AP-73D3 with BSA-PTX-MCC solid phase coating antigen was shown to be suitable for determining palytoxin concentrations in "spiked" pooled human plasma.
- A prototype simple, rapid latex agglutination test for detecting palytoxin was developed.
- The indirect CIEIA was validated against a mouse bioassay and a cytotoxicity assay, which we had previously developed, using coded "unknown" palytoxin samples
- MAb 73D3 was shown to be capable of neutralizing <u>in vitro</u> palytoxin induced cytotoxicity. Dr. Hewetson at USMRIID extended this observation and demonstrated <u>in vivo</u> neutralization of palytoxin lethality in mice.

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).

TABLE OF CONTENTS

LIST OF APPENDICES

LIST OF FIGURES

LIST OF TABLES

LIST OF SCHEMES

STATEMENT OF THE PROBLEM UNDER STUDY

Palytoxin is an extremely toxic non-proteinaceous toxin first isolated from marine soft corals of the genus *Palythoa*. It is a membrane active agent whose mechanism of action is not fully understood. Palytoxin is also a novel type of tumor promoter. Until recently, there was no specific means of detecting and quantitating palytoxin and there was no known antidote.

This project was undertaken in response to the U.S. Army's need for research quantities of palytoxin and a rapid, simple, and specific means to identify and quantitate palytoxin. The overall goals of this BAA project, as stated in the original proposal, were:

(i) to collect *Palythoa* soft coral, and isolate and purify specified quantities of palytoxin for delivery to USAMRIID,

(ii) to produce high affinity polyclonal and monoclonal antibody preparations against palytoxin hapten-protein conjugates, and

(iii) to develop sensitive immunoassays for detecting and quantitating palytoxin in biological samples.

The reagents and protocols needed for these immunoassays were to be delivered to USAMRIID. The contract was later ammended with the addition of two change orders as follows:

(iv) The isolation and purification of lyngbyatoxin A from *Lyngbya majuscula*, delivery of this toxin plus a further quantity of palytoxin to USAMRIID, and

(v) Investigation of the preparation of tritium labeled palytoxin, if successful, delivery of 10 to 15 mg of high specific activity [^{3}H]-palytoxin and any excess palytoxin isolated for the contract extension which we did not use.

BACKGROUND

Palytoxin is reputed to be the most potent non-proteinaceous toxin known [1,2]. It is a relatively low molecular weight (2678.5 g/mol) marine natural product isolated from tropical coelenterates of the genus, *Palythoa* [3]. The toxicity of *Palythoa toxica* was apparently recognized first by the ancient Hawaiians, whose leaders designated special warriors to smear the coral's mucinous exudate on spear-tips prior to combat [4]. In modern times, this legend enabled researchers at the University of Hawaii to rediscover the single set of tidepools containing *Palythoa toxica* near Hana, on the island of Maui.

The toxin was first isolated by University of Hawaii researchers, Moore and Scheuer in 1971 [5]. The chemical structure of palytoxin (Figure 1) was reported by Moore, and independently by Uemura and coworkers, in 1981 [6,7]. The same

toxin was also identified, although at a much lower concentration, in the far more abundant species, *Palythoa tuberculosa* [3]. Palytoxin is not composed of repeating carbohydrate or amino acid subunits, and has provided a unique challenge to natural products chemists attempting to isolate and characterize it. Kishi and coworkers, at Harvard University, have recently reported the total synthesis of a closely related compound, palytoxin carboxylic acid [8]. This example was cited by *Science* as the most complex chemical synthesis yet achieved, since palytoxin has 64 chiral centers and 42 hydroxyl groups [9].

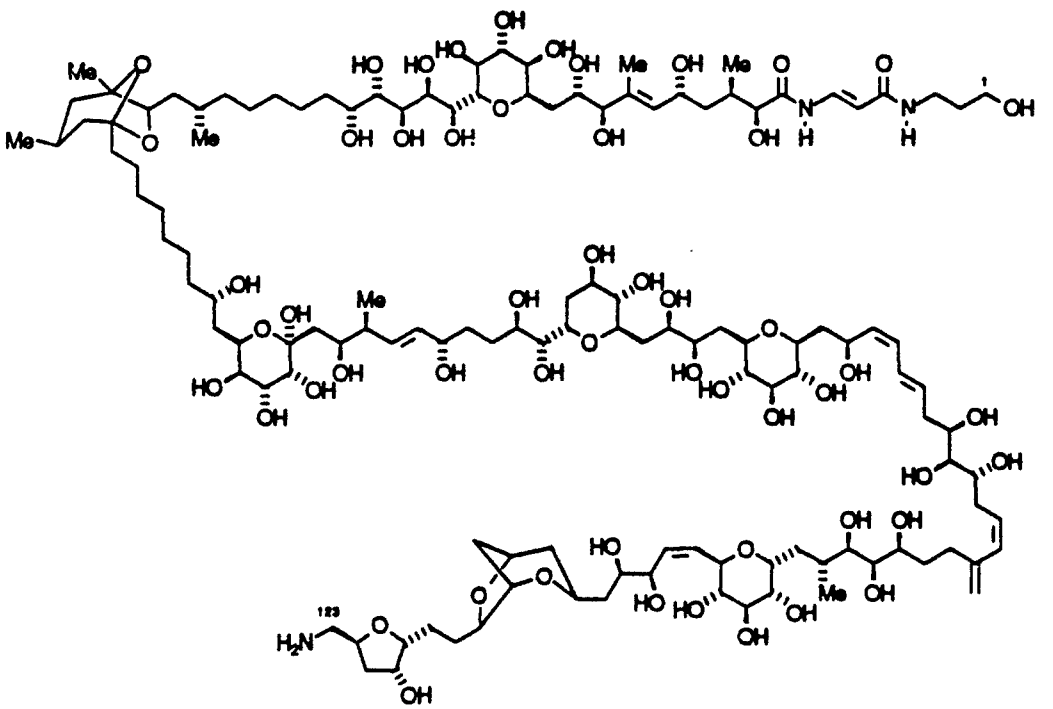


FIGURE 1. The structure of palytoxin.

The intravenous LD_{50} of palytoxin in the rabbit and mouse is 33 ng/kg and 450 ng/kg, respectively [10]. Palytoxin is a fast acting toxin which causes neurological and vascular distress in experimental animals, followed by death due to congestive heart failure [11]. At the cellular level, palytoxin is known to cause hemolysis [12], stimulate arachidonic acid metabolism [13] and alter transmembrane ion flux [14]. It is also a non-phorbol ester type of tumor promoter in the two stage model of carcinogenesis on mouse skin [15,16].

For many years, the only method available for identifying palytoxin involved the tedious procedures of mouse bioassay and purification followed by characterization of the compound by UV and NMR spectroscopy [3] Recently, HPLC methods have been reported [17].

During the SBIR project (Grant # 5R44AI20802) entitled "Development of Palytoxin Immunotoxins", funded by the Department of Health and Human Services, Hawaii Biotechnology Group, Inc. successfully developed an *in vitro* cytotoxicity (IVCTT) assay for determining the toxicity of samples suspected of containing palytoxin (PTX). This assay system measured the uptake of ^{14}C-leucine by murine T-cell leukemia EL-4 cells, cultured in a 96-well tissue culture plate in the presence and absence of test sample. As a method for determining the concentration of PTX in samples, the IVCTT assay has a detection limit for toxin of 5 pg/ml sample, but the system suffers from the following disadvantages:

(i) the system is inconvenient, as it requires aseptic technique, sterile glass and plasticware, and takes almost 24 hours from start to result,

(ii) The system requires the use of radioisotope, a laboratory licensed for the use of ^{14}C, and a scintillation counter,

(iii) the system requires availability of living EL-4 cells

(iv) the system does not measure PTX concentrations alone, as it cannot differentiate between toxicity due to PTX and effects due to other toxic molecules.

A more convenient system was therefore required that did not suffer from these disadvantages.

This BAA project was therefore proposed to develop sensitive and specific immunoassay systems for detection and quantitation of PTX in biological samples. Initially, this required synthesis of immunogenic conjugates of PTX coupled to keyhole limpet hemocyanin (KLH - to hyperimmunize animals for antibody production), and to bovine serum albumin (BSA - for use as a solid-phase coating antigen for immunoassay development with the antibodies produced against KLH-PTX).

The potential for success in this project was strengthened by reports that antibodies and/or immunoassays for several other important marine and aquatic toxins have been produced [19-23]. In 1986 we generated rabbit antisera to palytoxin (unpublished information). More recently, Levine and associates [24] reported the production of rabbit anti-palytoxin antibodies which were capable of neutralizing some of the biological properties of palytoxin.

MATERIALS AND METHODS

Collection of *Palythoa* spp.

Scuba gear was used to collect *P. tuberculosa* from vertical rock faces at depths of two to ten meters. One diver could harvest two to three kg of soft coral per tank of air. After collection, the material was immediately placed in sturdy plastic bags and packed in ice. Usually one day would elapse before the samples could be delivered to the laboratory. Upon receipt in the laboratory the bags of coral were weighed, and stored frozen at -20 °C.

P. Toxica was collected by scraping the animal from the rocks and walls of the tidepool with a knife. The polyps were collected with a small fishnet and immediately placed into a tared container of 95% ethanol. The container was tightly closed and packed on ice for transport to the laboratory. Isolation would commence within 24 hours.

Isolation of Palytoxin.

Palytoxin isolation followed the basic procedure, with some modifications, published by Moore [5]. We prepared a detailed instruction manual of isolation procedures for use by our isolation technicians. A copy of this HBG manual has been included as Appendix BB.

Synthesis of Haptens.

Protocols for the preparation of PTX-PDP and PTX-MCC are given in Appendices A and B, respectively.

Hapten-Carrier Conjugation.

The protocols used for preparation of KLH-PTX-PDP, BSA-PTX-PDP, KLH-PTX-MCC, BSA-PTX-MCC, and BSA-PTX are included as Appendices C-G, respectively.

Palytoxin-protein conjugates were characterized by an indirect method for the estimation of the moles of palytoxin per mole of protein. Total protein concentration was determined using the BCA protein analysis obtained from Pierce Chemical Co. (see Appendix H). Free thiol groups on the proteins were determined using the dithiodipyridine (DTDP) method (see Appendix I). The decrease in free sulfhydryl groups of thiolated proteins after reaction with the palytoxin haptens provides a means of estimating the extent of palytoxin conjugation to the carrier.

Extraction of *Palythoa* Coral.

Crude extracts of *Palythoa tuberculosa* were prepared by soaking approximately 10 grams of sliced, weighed soft coral in two volumes of 70% aqueous ethanol for 18 to 24 hours at room temperature. The extracts were harvested, centrifuged and stored at 4 °C. Over a period of weeks, precipitated material was

observed in some of the extracts. When it was observed, the precipitate was removed by centrifugation.

Immunization of Rabbits and Mice.

Rabbits were immunized according to standard procedures [25]. Primary immunization was with 400 to 500 ug KLH-PTX-PDP in 1 ml phosphate buffered saline, pH 7.2, and 2 ml complete Freund's adjuvant. Secondary boosts utilizing 250 ug of immunogen in incomplete Freund's adjuvant were given one to four months later. All rabbit immunizations were divided among subscapular and intramuscular sites. Animals were bled by cardiac puncture under general anesthetic.

Mice were also immunized in a standard fashion [26-28]. Primary innocula contained 100 ug of immunogen in approximately 0.3 ml complete Freund's adjuvant. Secondary innocula consisted of 50 ug of immunogen in incomplete Freund's adjuvant. All injections were administered intraperitoneally. Mice were bled from their tail vein to obtain sera for testing their response to immunization. Mice to be used as spleen cell donors for hybridoma production were given 50 ug immunogen in 0.2 ml phosphate buffered saline, pH 7.2, by the intraperitoneal route, four days prior to sacrifice.

Hybridoma Generation and Monoclonal Antibody Production.

Standard procedures [26-28] were followed using P3X63Ag8.653 as the fusion partner.

Primary Indirect ELISA Screen For Polyclonal and Monoclonal Antibodies.

The protocol for the primary ELISA screen for rabbit or mouse antibodies to palytoxin is presented in Appendix J.

Purification of Monoclonal Antibodies.

Throughout this project, IgG antibodies were purified from mouse ascites fluid by protein G chromatography (Genex) using the following procedure. Clarified ascites fluid was microfuged to remove particulate matter then diluted 1:1 with column buffer (0.01 M sodium phosphate, pH 7.0, 0.15 M sodium chloride). The diluted ascites fluid was layered onto a protein G column (1 ml) and washed into the column with column buffer at 1 ml/minute flow rate. Non-bound proteins in the effluent were monitored by UV absorbance at 280 nm. Once the non-IgG fractions had washed through the column, as determined by a return to baseline UV absorbance, the IgG fraction was eluted with 0.5 M ammonium acetate, pH 3.0. Fractions containing eluted IgG were immediately neutralized with 1.5 M tris-HCl, pH 8.8. IgG containing fractions were then combined and dialyzed extensively against PBS, pH 7.0.

Affinity purification of IgG from mouse ascitic fluid and ammonium sulfate precipitated rabbit immunoglobulins using Protein A-Sepharose was performed using the protocol in Appendix K.

Biotinylation of MAb 73D3.

Monoclonal antibody 73D3 was biotinylated using a modification of the procedure described by Goding [28]. Briefly, 440 µl of 73D3 (1.0 mg/ml, 293 nmoles) in sodium carbonate buffer pH 8.0-8.3 was reacted with 22.8 µl 10 mM sulfo-NHS-biotin (Pierce Chemical Co.) in 1.0 M sodium carbonate buffer for four hours at room temperature. The product was dialyzed four times against 100 volumes PBS, pH 7.0 and the protein concentration determined by UV spectroscopy.

Alkaline Phosphatase Conjugates.

Alkaline Phosphatase (AP) was conjugated to Protein A-purified R7B7 immunoglobulins using a modification of the method of Avrameas [29,30], and the procedure detailed in Appendix L (which was also employed for MAb 73D3). AP was conjugated to Protein A-purified 73D3 MAb and PTX-MCC hapten using the procedures detailed in Appendices L and M respectively. Methods for determination of free sulfhydryl groups and total protein concentration are given in Appendices I and H respectively.

Enzyme Immunoassays for Detection and Quantitation of Palytoxin.

During Year 2 -4, the palytoxin CIEIA and sandwich ELISA procedures were carried out according to the protocols presented in Appendices N-R, except that standard or unknown palytoxin samples used in CIEIA were diluted in PBS containing 1% BSA and 0.05 mM $Na_2B_4O_7$. The inclusion of the borate was previously found to improve the performance of the palytoxin sandwich ELISA and had no effect on the palytoxin CIEIA.

Indirect Competitive Inhibition Enzyme Immunoassay (Indirect CIEIA): The protocol for the palytoxin indirect CIEIA using rabbit antisera or immunoglobulin fractions, or mouse polyclonal or monoclonal antibody preparations, is included as Appendix N.

Indirect Sandwich ELISA:. The protocol for the indirect sandwich ELISA for palytoxin is included as Appendix O.

Direct CIEIA using AP-73D3 conjugate: The protocol for performing the direct CIEIA using AP-73D3 conjugate is given in Appendix P.

Direct CIEIA using AP-PTX conjugate: The protocol for performing the direct CIEIA using AP-PTX conjugate is given in Appendix Q.

Direct Sandwich ELISA using AP-R7B7 conjugate: The protocol for the direct sandwich ELISA using AP-R7B7 conjugate is given as Appendix R.

Palytoxin Indirect CIEIA Specificity Determination.

Compounds to be compared in defining the specificity of the palytoxin CIEIA were diluted in PBS containing 1% BSA and 0.05 mM $Na_2B_4O_7$ and treated identically with diluted samples of palytoxin. Stock solutions of some compounds had to be dissolved in ethanol

or DMSO because of lack of water solubility. These stocks were diluted greater than 100-fold into buffer and the highest concentrations of organic solvents thus obtained were tested and found not to interfere with immunoassay results.

Antibody Neutralization of *in vitro* Palytoxin Cytotoxicity.

The method for studying the *in vitro* cytotoxicity of palytoxin on EL-4 cells using uptake of ^{14}C-L-leucine is given in Appendix S. Purified palytoxin was stored in 50% aqueous ethanol and subjected to an initial dilution of 100-fold, or greater. All palytoxin and antibody dilutions were performed in leucine-free MEM supplemented with 10% calf serum. Protocols for the addition of palytoxin, antibody and cells varied with experimental design and are indicated with the experimental results.

Chemicals.

All chemicals and biochemicals were purchased from reliable commercial sources. Solvents used were Alrich anhydrous grade.

Chromatography.

High Performance Thin Layer Chromatography plates (HPTLC NH_2 F_{254} #15647 and HPTLC silica gel F_{254} #13727) were purchased from EM Science. Two solvent systems were routinely used:

NH_2 plates - 9:8:6 pyridine-water-*n*-amyl alcohol
silica plates - 9:6:7 pyridine-water-*n*-amyl alcohol

Two HPLC methods were utilized:

Method A: Zorbax ODS column (4.6 X 250 mm), 40% acetonitrile-0.05 N acetic acid, 1 ml/min, 263 nm.
Method B: Showdex OHpak B804 column (8 X 500 mm), 10:1 0.02 N phosphate buffer (pH 4.6)-ethanol, 1 ml/min, 263 nm.

Data Analysis.

Each data point was usually done in triplicate and the mean value calculated. Plates usually contained larger numbers of wells containing no inhibitor in order to give a better estimate of the maximum response (B_O). B/B_O values were calculated as follows: the mean of a given set of replicates was divided by the mean of all the wells containing no inhibitor. For determination of unknowns, the Rodbard four parameter logistic function [31], as found in the BioRad MacReader program for the Macintosh computer was used. Appropriate statistics were calculated as needed.

Isolation of Lyngbyatoxin A.

Lyngbyatoxin A was isolated by the published procedure of Moore and Cardelina [32,33]. The toxin was characterized by HPLC and ^{1}H-NMR.

RESULTS

PALYTOXIN PRODUCTION

Palythoa Collection

Palytoxin was isolated from two closely related species of soft coral, *Palythoa toxica* and *P. tuberculosa*. *Palythoa toxica* was collected from the Hana, Maui tidepool originally described by Moore, Helfrich and Patterson [3,5]. This tidepool is quite small and contains a very limited amount of *P. toxica*, therefore, the more abundant species, *P. tuberculosa* was used as the source for the majority of the toxin. Unfortunately, the concentration of palytoxin in *P. tuberculosa* is much less than that in *P. toxica*. Two scouting and collecting trips to the south and north shores of Maui yielded only minimal amounts of *P. tuberculosa*. This coral was much more abundant and of better quality at Okala Island, a remote collecting site off of Moloka'i which had been previously identified. Arrangements were made with a local boat operator to transport parties of two to four divers to the north shore of Moloka'i between Kalaupapa Peninsula and Halawa Valley.

The condition of the *P. toxica* in the "shark" tidepool at Hana, Maui was monitored periodically by our staff and local contacts on Maui. Collection of the organism were performed only when more than one half of the available submerged substrate was covered with healthy appearing *P. toxica*. This precaution was taken so as not to endanger this unique organism, which has not been reported at any other location in the world. We do not know whether variations in the growth were due to natural conditions or interference by man. Subjectively, there appeared to be natural fluctuations in the coral growth. Table 1 summarizes the collections of *P. toxica* which were completed during this project.

TABLE 1. Collection of *Palythoa toxica* from tidepool at Hana, Maui.

Date	Weight (g)	Comments
7/15/87	120	Coral appeared healthy; > 60% of submerged rocks were covered with growth
12/03/87	0	Very sparse growth; no collection made
2/8/89	150	Coral appeared healthy; > 60% of submerged rocks were covered with growth
4/16/90	50	Small collection; coral did not look healthy; ≤ 50% of submerged rocks were covered with growth

April 16, 1990 was selected for *P. toxica* collection due to the extremely low tide at noon. Because of the low tide, a survey of other tide pools in the area was possible. At least two other pools contained *P. toxica*-like growth, which was sampled. Seventy percent ethanol crude extracts from at least three of these samples exhibited high activity in CIEIA and <u>in vitro</u> cytotoxicity assays for palytoxin. This indicates that *P. toxica* containing

potent palytoxin is present in tide pools at Hana other than the "shark" pool .

A total of 523.53 kg of P.tuberculosa was collected during sixteen expeditions undertaken during the summer months. These collections are summarized in Table 2. Regrowth of this species was fast and no reduction in the overall growth was evident from one year to the next.

TABLE 2. Summary of *Palythoa Tuberculosa* Collection

Bag #'s	Collection Site	Date	Weight (kg)
1–10	Nuu Bay, Maui	6/12/87	33.37
11–15	Keawenui, Moloka'i	6/28/87	14.68
16–24	Okala Island, Moloka'i	6/29/87	27.86
25–31	Maliko, Maui	7/25/87	21.72
32–43	Okala Island, Moloka'i	8/02/87	40.42
44–58	Okala Island, Moloka'i	8/15/87	51.16
59–69	Okala Island, Moloka'i	9/05/87	38.81
		1987 Subtotal	228.02
70–79	Okala Island, Moloka'i	6/29/88	30.05
80–85	Okala Island, Moloka'i	8/02/88	18.44
86–97	Okala Island, Moloka'i	8/16/88	35.46
98–105	Okala Island, Moloka'i	9/30/88	21.37
		1988 Subtotal	105.32
106–115	Okala Island, Moloka'i	6/13/89	36.76
116–127	Okala Island, Moloka'i	7/26/89	44.66
128–138	Okala Island, Moloka'i	8/16/89	39.23
139–147	Okala Island, Moloka'i	9/16/89	28.46
		1989 Subtotal	149.11
148–159	Okala Island, Moloka'i	8/18/90	41.08
		1990 Subtotal	41.08
		Total	523.53

The bags of *P. tuberculosa* which were used in each isolation are listed below. Bags #1-15 had to be discarded due to failure of the freezer on the night of July 15, 1987. Bags # 25-31, from Maliko, Maui, were discarded when small scale isolations of this material failed to yield any palytoxin.

Palytoxin Isolation

Palytoxin was isolated and purified via a procedure based on that published by Moore [5]. A flow diagram of the procedure followed is presented in Figure 2.

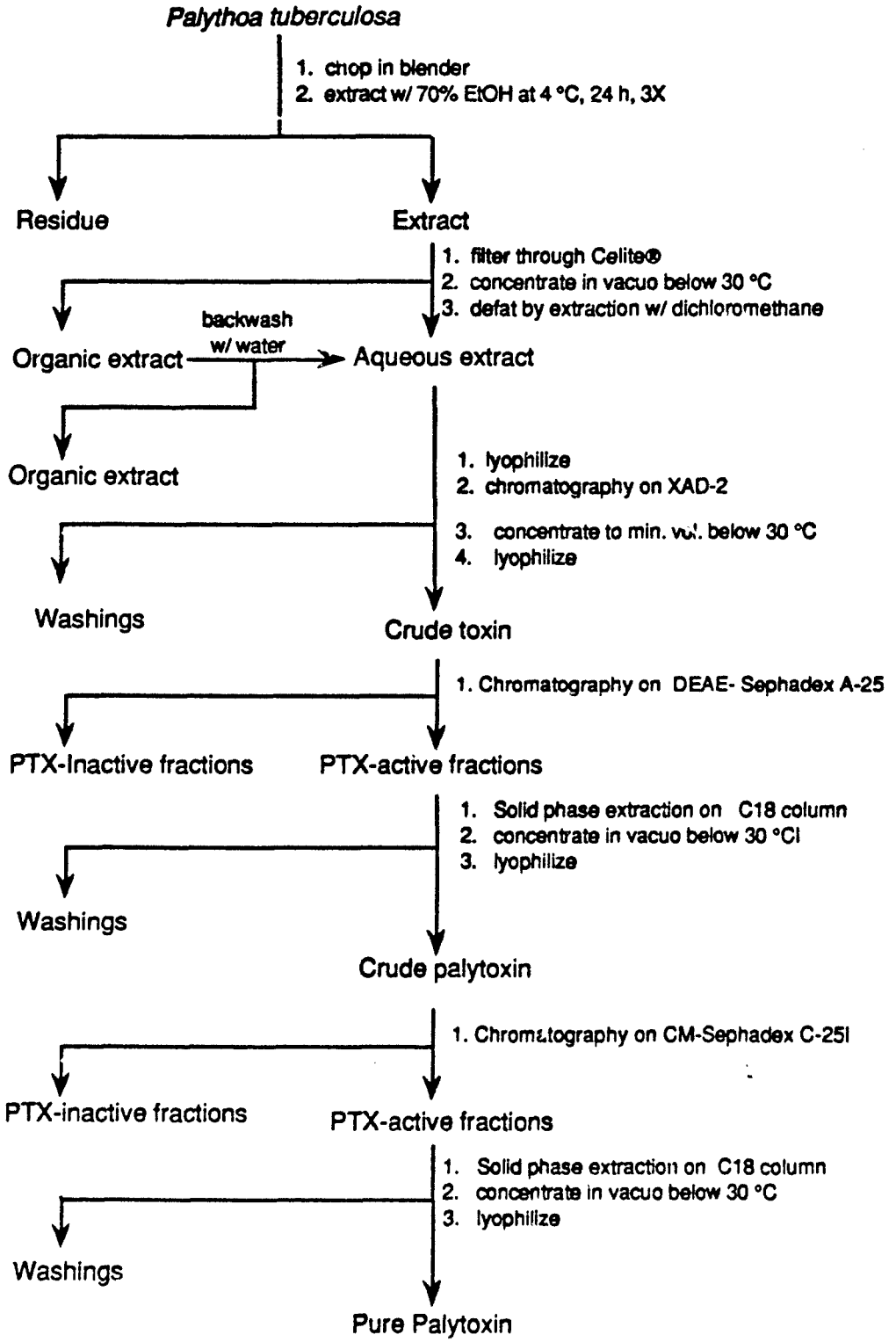


FIGURE 2. Isolation of Palytoxin.

HBG had an inventory of approximately 300 mg of palytoxin, which had been isolated from *P. toxica*, prior to the beginning of this contract. The large scale isolation laboratory was not fully installed and operational until December 1987, therefore, we borrowed from our pre-existing toxin supply as necessary for hapten synthesis. Prior to the arrival of the large rotary evaporator and other equipment needed for scaling up the isolation, a series of small isolations from *P.tuberculosa* provided us with an additional 50 mg of palytoxin. Table 3 summarizes the isolation experiments performed following the establishment of an isolation laboratory. Each batch of palytoxin was characterized by UV spectroscopy and judged to be greater than 95% pure. Beginning with the second year's shipment, HPLC analysis [34,35] was adopted as an additional criterion of purity.

Inspection of this data illustrates the great difference in toxin content of the two *Palythoa* species. The % yield of toxin from *P.toxica* ranged from 8.00-27.83%, whereas the yields for *P. tuberculosa* ranged from 0-0.54%. The data also reveals considerable experiment to experiment variation in yields. There is no apparent correlation of yield with the age of the coral specimen.

The low yield from *P. toxica* in experiment 4.2 was the result of a modification in the procedure designed to isolate palytoxin carboxylic acid. This experiment is described in the section on the synthesis of non-amino derivatized haptens of this report. Approximately 4.5 mg of palytoxin of unacceptable purity was also isolated from this experiment and added to the material in isolation 4.3. Thus the true yield of palytoxin from isolation 4.2 was probably closer to 16%.

Table 3 also demonstrates that the yield from *P. tuberculosa* was significantly improved over the course of this project. Average yields for each year were: Year 1 - 0.16%, Year 2 - 0.11%, Year 3 - 0.23%, and Year 4 - 0.26%. Most striking are the vastly improved yields for the last three isolations, 4.6-4.8. The average yield for these three experiments was 0.41%, a 3-fold increase over our initial yields.

This procedure underwent extensive modification and refinement during this project. Attempts to improve the process during the first 2 years were frustrated by the lack of a fast, sensitive assay. Efforts to utilize the early indirect CIEIA (see section on immunoassay development) were frustrated by its poor reproducibility. These experiments indicated that only 2-5% of the toxin in the crude extracts was being recovered. Once the direct CIEIA was developed, it proved to be very useful for optimization of the isolation procedure.

In the early part of this work, isolation experiments were conducted start-to-finish without a break, as we had been told by Moore that this was necessary for good yields. In mid-1988, we began to store extracts and column fractions overnight at 4 °C and found that there was no obvious effect on yields. In early 1989, we acquired a cold room in which to do the extraction and chromatography of palytoxin. The average yields for 1989 were ~2X those of the previous two years.

TABLE 3. Summary of Palytoxin Isolation.

Experiment	Date	Species	Bag #'s	Weight (kg)	Yield (mg)	% Yield
1.1	7/16/87	*P. toxica*		0.120	33.4	27.83
1.2	8/14/87	*P. tuberculosa*	20, 24	6.55	3.8	0.06
1.3	9/2/87	*P. tuberculosa*	16, 17, 18, 19	11.35	15.9	0.14
1.4	12/11/87	*P. tuberculosa*	21, 22, 23, 34	12.46	17.1	0.14
1.5	2/17/88	*P. tuberculosa*	32, 33, 36	9.83	27.8	0.28
				year 1 subtotal	97.9	
2.1[a]	4/4–4/19/88	*P. tuberculosa*	40, 41, 43	8.05	14.7	0.18
2.2	5/12–5/22/88	*P. tuberculosa*	44, 45, 46	10.01	13.8	0.14
2.3	6/13–6/28/88	*P. tuberculosa*	47, 49, 50	9.83	5.2	0.05
2.4[b]	7/11–7/29/88	*P. tuberculosa*	70, 71, 72, 74	10.99	-	0.00
2.5[b]	8/8–8/29/88	*P. tuberculosa*	73, 76, 77, 79	10.34	-	0.00
2.6	9/19–10/7/88	*P. tuberculosa*	75, 78, 81	9.79	16.2	0.17
2.7	10/17–11/1/88	*P. tuberculosa*	48, 51, 53	10.30	17.0	0.17
2.8[c]	11/1--11/14/88	*P. tuberculosa*	82, 83, 84	10.00	6.7	0.07
2.9	11/14–11/30/88	*P. tuberculosa*	85. 86. 87	9.90	16.2	0.16
2.10	12/12–12/28/88	*P. tuberculosa*	102, 103, 104, 105	10.06	15.0	0.15
2.11	2/9–2/22/89	*P. toxica*		0.15	24.7	16.47
				year 2 subtotal	129.5	
3.1[d]	2/27–3/21/89	*P. tuberculosa*	93, 94, 95	9.79	22.5	0.23
3.2	3/27–4/13/89	*P. tuberculosa*	96, 97, 98, 99	9.28	15.5	0.17
3.3[e]	4/24–5/26/89	*P. tuberculosa*	88, 89, 100, 101	10.84	58.2	0.54
3.4[f]	7/17–8/10/89	*P. tuberculosa*	106, 107, 108	9.58	0.0	0.00

TABLE 3. Continued

3.5	8/14–9/18/89	*P. tuberculosa*	65, 80, 90, 91, 92	10.01	19.5	0.19
3.6	10/2–10/31/89	*P. tuberculosa*	37, 42, 52	10.20	23.2	0.23
3.7[g]	11/13–12/21/89	*P. tuberculosa*	54, 55, 56, 57	10.50	(15.6)	
3.8[g]	1/2–2/5/90	*P. tuberculosa*	109, 110, 111	9.47	(6.8)	
3.9[h]	2/8–2/9/90	*P. tuberculosa*		-----	16.7	
3.10[g]	2/12–2/27/90[g]	*P. tuberculosa*	113, 114, 115	10.47	(12.4)	
				year 3 subtotal	155.6	
4.1[i]	3/13–6/7/90	*P. tuberculosa*	58, 59, 60	10.24	18.8	0.18
4.2[j]	4/17–5/3/90	*P. toxica*		.05	4.0	8.00
4.3	5/29–6/29/90	*P. tuberculosa*	122, 124, 142	10.40	11.8	0.11
4.4[k]	7/17–9/25/90	*P. tuberculosa*	125, 141, 145	10.55	24.0	0.23
4.5[l]	10/18–12/30/90	*P. tuberculosa*	62, 120, 143	10.80	5.9	0.05
4.6[m]	1/2–2/1/91	*P. tuberculosa*	69, 112, 131	12.08	50.0	0.41
4.7	1/24–2/15/91	*P. tuberculosa*	63, 66, 127, 132, 139	18.00	92.2	0.51
4.8	2/12–3/25/91	*P. tuberculosa*	118, 150, 151, 155, 159, (+ one unmarked bag from pre-contract work)	19.30	60.3	0.31
				year 4 subtotal	267.0	
				Project Total	650.00	

a Attempted defatting step by continuous extraction with CH_2Cl_2.

b On 7/11/88 we started using 60 cc. C-18 mega BondElut columns after the DEAE and CM columns. UV spectrum of the final product showed no palytoxin--it was probably lost on the BondElut column. The same problem occurred on 8/8/88. This was not rectified until the isolation on 9/19/88 when we used a larger (ca. 1000 ml) eluting volume of 80% aqueous ethanol.

c The yield decreased on 11/1/88 when we used a regenerated CM-Sephadex column. Using new column material in both the CM and DEAE columns improved our yields in succeeding isolations.
d Started perfoeming extraction of coral and chromatography in cold room.
e Unusually high yield of very pure toxin; best isolation to date; reason for high yield is unknown.
f Mistake by new technician resulted in oecomposition of toxin during final step of isolation.
g After 3 repetitions of the CM-Sephdex column, the toxin was of unacceptable purity; combined with impure fractions from earlier isolations; not included in total below.
h CM-Sephadex purification of combined impure toxin fractions from earlier isolations; final purity was acceptable.
i Technician unexpectedly left 3/19/90; crude material stored in freezer until 5/7/90
j Modified procedure designed to isolate PTX-COOH see section on alternative haptens for details.
k Initial yield 12 mg but CIEIA indicated large loss in defatting step; ch2cl2 evaporated and chromatographed to yield additional 12 mg
l Tried to eliminate defatting extraction
m Began using blender to prepare coral for extraction

One of the most time-consuming and difficult steps of the procedure is the defatting via CH_2Cl_2 extraction. Extremely thick and stable emulsions are frequently formed in this extraction. Moore used a mixture of benzene and *n*-butanol for the defatting [5]. In our hands, this procedure offered no advantages over CH_2Cl_2 extraction, stable emulsions were still formed. In addition, the amount of benzene required presented additional safety concerns. Chloroform extraction also proved to be no better than CH_2Cl_2 extraction. To reduce the emulsion formed, we decided to investigate the use of continuous liquid-liquid extraction with CH_2Cl_2 (isolation # 2.1). This proved to be even more time-consuming with no improvement in the yield of toxin.

Early in the project, Moore informed us that palytoxin was unstable to storage in phosphate buffer and that desalting must be performed immediately following both the DEAE and CM columns. Initially, the column effluants were concentrated on the rotovap and then applied to a 1 ml C-18 Bondelut SPE column. In 1988, Analytichem introduced the Mega Bondelut SPE columns. Using a larger size SPE column (60 ml following the DEAE; 20 ml following the CM) equipped with a vacuum feed allowed us to apply the entire effluant volume to the SPE column and avoid extended exposure of the toxin to phosphate buffer. Initial results were disappointing (experiments 2.4 and 2.5) until we determined that the volume of aqueous ethanol required to elute palytoxin from the C-18 did not scale up in a linear fashion from the 1 ml SPE column. This innovation is also credited with the 2-fold improvement in yields for 1989.

In 1990 we again attempted to determine the overall efficiency of our PTX isolation. Samples of the fractions at each stage of isolation 4.4 were saved and tested by both direct CIEIA and cytotoxicity. The results indicate that we experienced a dramatic loss of PTX during the defatting step. Both the CIEIA and IVCTT assays indicated that the initial crude extract contained ~1 g of palytoxin. We obtained only 12 mg of purified toxin at the end of our normal procedure. The assays indicated

that the aqueous effluent from the XAD column still contained 65 mg of PTX. Additional toxin was detected in the CH_2Cl_2 from the defatting step of that isolation. These fractions were concentrated and processed to yield an additional 12 mg of pure PTX.

In an attempt to account for the losses experienced in the defatting step, we performed two experiments where a known amount of PTX in water was extracted with CH_2Cl_2 and the PTX was measured by CIEIA and UV in both layers and the interface. The results of the two assays do not agree and the total palytoxin in the three fractions did not equal the amount we started with. CIEIA indicates that 36% of the toxin was recovered while UV indicates a 67% recovery. As a corollary to these solvent partitioning experiments, we looked at the reproducibility of measuring PTX concentrations by UV. Two different samples were quantitated 10X over a 1 week period. The reproducibility was shown to be very good (Sample 1 - mean: 3.604 ± 1.5% S.D. & Sample 2 - mean: 2.373 ± 2.8% S.D. One possible explanation for these observations could be that palytoxin, because it has both hydrophilic and hydrophobic regions, could form micelles with CH_2Cl_2 in water and reversed micelles with water in CH_2Cl_2. This explanation could account for the persistance of emulsions formed in the defatting step and the presence of palytoxin in the CH_2Cl_2, a solvent in which palytoxin is insoluble. This hypothesis could also account for the discrepancy between the amount of palytoin measured by CIEIA and UV in these solvent partitioning experiments. An aggregate of palytoxin, such as a micelle, may not react in a 1:1 manner with the antibody and therefore one would obtain an erroneously low result.

Because of this evidence that significant quantities of PTX are lost during the defatting procedure, we decided to eliminate this step from the next isolation (4.5). Unfortunately, this complicated further processing of the extract. The solution was very thick and viscous and plugged the DEAE column. The gel had to be extruded from the column and extracted with several liters of aqueous ethanol in a Büchner funnel to recover the toxin. Following several days of concentration, the material was gel filtered on LH-20 in an attempt to remove some of the sludge. The resulting concentrate was not as thick and viscous as before and was successfully run through another DEAE column. The final yield from this difficult isolation was only 5.9 mg. Therefore the defatting step was reinstated in the procedure.

We then adopted the direct CIEIA as a standard tool for monitoring each step of the isolation, much as a synthetic chemist uses TLC. If a large drop in the amount of toxin was observed after a completed step, all fractions from that step would be recovered and resubjected to the procedure. With this as a tool, the yields of the final three isolations (which also made use of a blender - see below) were dramatically higher.

The initial preparation of the coral for extraction involved chopping it into thin slices with a cleaver. There are obvious safety hazards to this step. Technicians could easily cut themselves and allow crude toxin to enter their bodies. In

addition, one must be concerned with possible formation of aerosals during this procedure. In 1987 and again in 1989, a food processor was evaluated for the initial chopping of the coral. This proved to be unworkable. In March 1989, the effect of lyophilizing and pulverizing *P. tuberculosa* prior to extraction was investigated. Successful execution of this procedure would have significantly reduced the toxin exposure hazard inherent in the isolation procedure. Additional advantages would have included reduced storage requirements for the raw material; the ability to freeze-dry coral on a continuous basis without the need for constant supervision; less water in the crude extracts due to concentration in the solid coral; less solvent required for extraction; and finally, more efficient extraction due to greater surface area. These factors were expected to result in improved throughput and possibly higher yields.

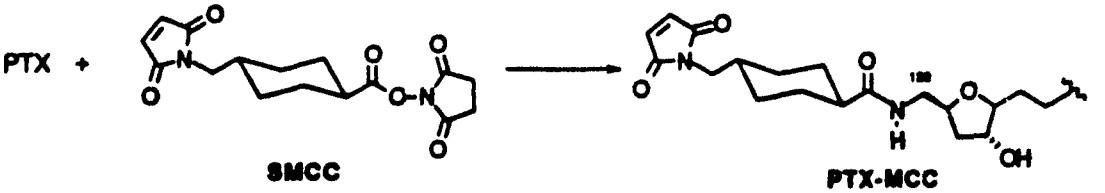
A large piece of coral was divided into halves and weighed. One half was freeze-dried and extracted, the other was treated in our conventional way. Following concentration, the extracts were redissolved so that the wet weight to solvent ratio was the same. Unfortunately, the results of these experiments showed that lyophilization of *P. tuberculosa* prior to extraction resulted in a 5-6 fold reduction of the antigenicity of extracted PTX as measured by direct CIEIA, and a 6-8 fold reduction in its biological activity (toxicity) as measured by in vitro cytotoxicity tests Appendix S).

In January 1990, a heavy duty blender became available to us. This was found to quickly pulverize the coral and resulted in a dramatic increase in the % recovery of palytoxin in isolations 4.6-4.8. CIEIA indicated the following percent recovery of pure palytoxin from the crude extract: #4.6 - 50.0/155 = 32.2%; #4.7 - 92.2/274 = 33.7%; #4.6 - 60.3/195 = 30.9%.

SYNTHESIS OF AMINO-DERIVATIZED PALYTOXIN—CARRIER PROTEIN CONJUGATES

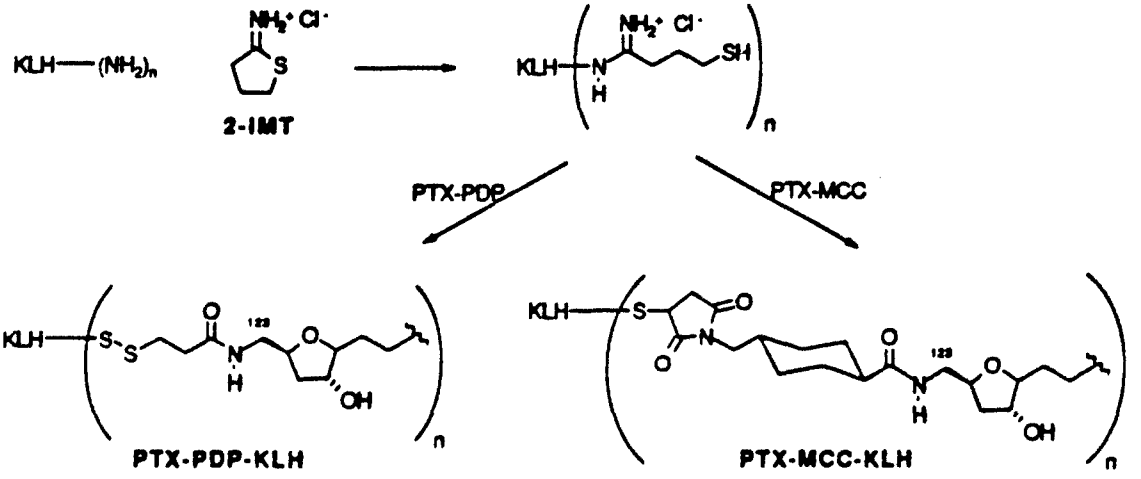
Palytoxin-carrier protein conjugates were prepared for use as immunogens or as coating antigens for immunoassay development. The synthesis of haptens for the conjugation of palytoxin via the C.123 amino group had been previously developed by us prior to the initiation of the present contract work. The syntheses of the two amino terminus haptens are presented in Schemes 1 and 2. Treatment of palytoxin with the commercially available bifunctional linkers, succinmidyl 3-(2-pyridyldithio)propionate (SPDP) [36] and succinimidyl 4-(N-maleimidomethyl)cyclo-hexane-1-carboxylate (Sulfo-SMCC) [37] yielded the corresponding amide derivatives PTX-PDP and PTX-MCC. Both haptens were characterized by ^{1}H NMR spectroscopy.

SCHEME 1. Synthesis of PTX-PDP.

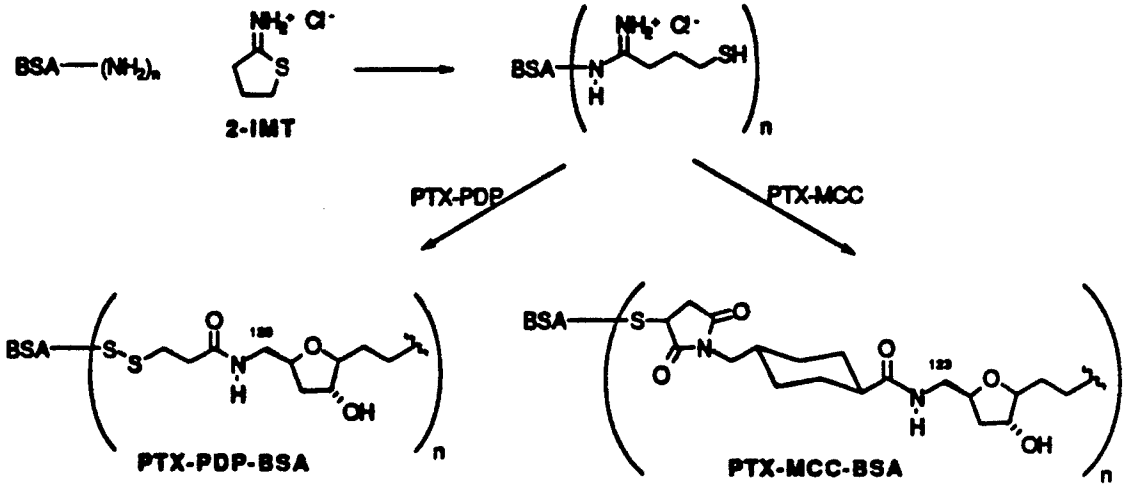


SCHEME 2. Synthesis of PTX-MCC.

PTX-PDP and PTX-MCC react with protein thiol residues to form disulfide bonds and thioether bonds, respectively. Two immunogens, KLH-PTX-PDP and KLH-PTX-MCC were prepared by addition of the corresponding hapten to protein which had been previously thiolated via 2-iminothiolane (2-IMT) treatment (Scheme 3). The ELISA coating antigens, BSA-PTX-PDP and BSA-PTX-MCC, were prepared in a similar manner from thiolated BSA (Scheme 4).



SCHEME 3. Synthesis of Immunogens.



SCHEME 4. Synthesis of ELISA Coating Antigens.

Palytoxin-protein conjugates were characterized by an indirect method for the estimation of the moles of palytoxin per mole of protein (see Methods Section). Typical conjugation ratios (moles PTX:moles protein) of ~8:1 were obtained for conjugation of either hapten to KLH and ~1.5 – 2.0:1 for conjugation of either hapten to BSA. Although an indirect method was used for characterization of the palytoxin-protein conjugates, the results obtained appear adequate to allow a reasonable level of standardization and reproducibility of the product.

DEVELOPMENT OF RABBIT POLYCLONAL ANTIBODY BASED IMMUNOASSAY

Production of Rabbit Polyclonal Antibodies.

Hyperimmunization of rabbit HBG 007 with KLH-PTX-PDP and identification of this animal as a source of useful anti-palytoxin antibodies had been achieved during an earlier DHSS SBIR project. However, this rabbit continued to provide a source of polyclonal antibodies throughout Years 1-3 of this BAA contract. Several different bleeds from this rabbit had been stored as ammonium sulfate precipitates at 4 °C for three to four years at HBG. During the course of the present contract, we established that the samples marked "Rabbit 7 bleed 7 (R7B7)" exhibited the highest titer of anti-PTX activity in the indirect sandwich ELISA systems (Appendix O).

Development of Enzyme Immunoassay using Rabbit Polyclonal Antibodies

Rabbit HBG 007 was bled two weeks after the second booster immunization and the serum tested by ELISA, using the protocol given in Appendix J, for the presence of antibodies against palytoxin. The results of this assay are shown in Figure 3.

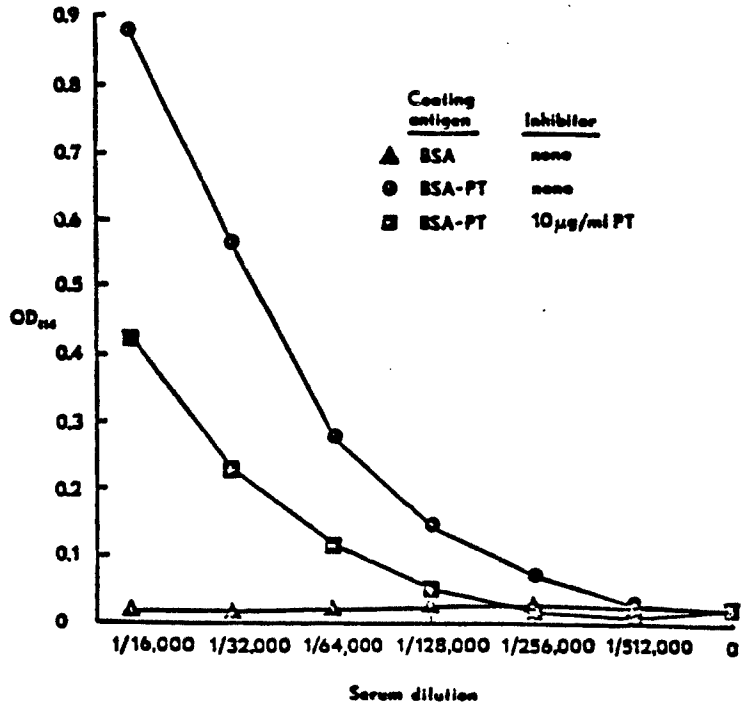


FIGURE 3. Primary ELISA Screen for Rabbit Antibodies to Palytoxin. The protocol given in Appendix J was followed. Rabbit 007, bleed 3 serum was subjected to serial dilution and tested on BSA and BSA-PTC-PDP coated microtiter plate wells in the presence or absence of free palytoxin. Absorbance at 414 nm (OD_{414}) of wells was measured one hour after substrate addition.

The presence of antibody against palytoxin hapten is shown by rabbit immunoglobulin binding to BSA-PTX-PDP, but not BSA alone. The palytoxin-specific nature of this binding is demonstrated by the fact that free palytoxin causes significant inhibition of this binding (Figure 4).

A checkerboard titration was performed to determine the optimal concentrations, for use in an indirect CIEIA, of BSA-PTX-PDP coating antigen (a saturating dose) and rabbit anti-palytoxin antibody (approximately one half saturation). Goat anti-rabbit alkaline phosphatase conjugate was used in this system at a dilution which gave an OD_{414} value of 0.3 to 0.8 after one hour of incubation with substrate at room temperature. Dilutions of palytoxin were tested in this optimized CIEIA to establish a standard curve. As shown in Figure 4, palytoxin inhibition decreased from about 50% to near zero over a range in palytoxin concentration of 10 ug/ml to 1 ng/ml.

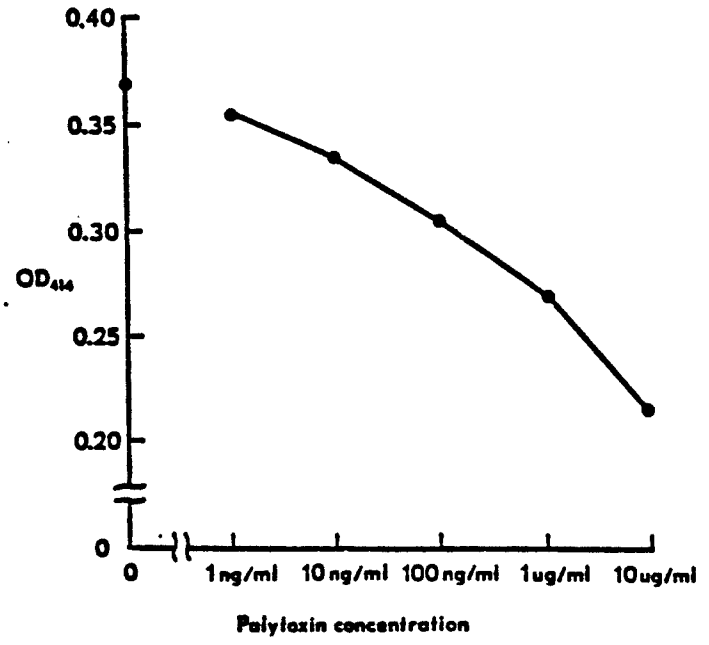


FIGURE 4. Titration of Palytoxin in Rabbit Polyclonal Antibody Indirect CIEIA. The protocol given in Appendix N was followed. Rabbit anti-palytoxin antiserum HBG 007, bleed 4 diluted 1/32,000 was used. Palytoxin was diluted as indicated. OD_{414} was determined 45 minutes after substrate addition.

Although the slope of the inhibition curve was shallow and incomplete, it could be used for the determination of palytoxin in unknown samples. When a new batch of BSA-PTX-PDP was prepared on March 12, 1987 and tested in the CIEIA system, full inhibition was seen over the same range of palytoxin concentrations (see Figure 5).

The steeper slope of the inhibition curves observed in Figure 5 resulted in full inhibition at a palytoxin concentration of 10 µg/ml. This result was encouraging since it offered the possiblity of more accurate measurements of palytoxin concentration in unknown samples. However, the completeness of inhibition at a palytoxin concentration of 10 µg/ml began to diminish over the succeding months. Subsequently, it was determined that the age of the coating antigen had an effect on the slope of the inhibition curve, as shown in Table 4.

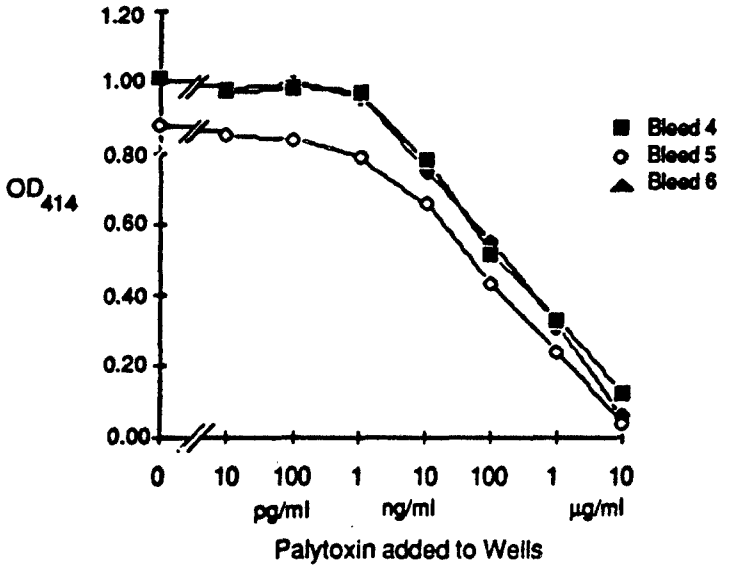


FIGURE 5. Titration of Palytoxin in Rabbit Polyclonal Antibody Indirect CIEIA using BSA-PTX-PDP coating antigen prepared March 2, 1987. The protocol given in Appendix N was followed. Rabbit antisera were diluted 1/16,000.

The suitability of BSA-PTX-MCC as a coating antigen for use with rabbit anti-palytoxin serum in the indirect CIEIA was evaluated. Figure 6 shows that the standard curves for the system using BSA-PTX-MCC coating antigen were very similar to those using aged BSA-PTX-PDP coating antigen. Thus, whereas rabbit anti-palytoxin serum was fully inhibitible in the indirect CIEIA using freshly prepared BSA-PTX-PDP coating antigen, only partial inhibition was observed when either BSA-PTX-MCC or aged BSA-PTX-PDP coating antigens were used.

TABLE 4. Effect of the Age of the BSA-PTX-PDP Coating Antigen on Inhibition of Rabbit Anti-palytoxin Antiserum HBG 007, Bleed 4 by Free Palytoxin.

	Batch of BSA-PTX-PDP prepared:	
	3/26/86	3/2/87
Date Tested	B/B_O at 10 ug/ml Palytoxin[a]	
5/06/87	0.33	0.05
7/01/87	0.50	0.16
8/05/87	n.d.[b]	0.27

a. B/B_O = OD_{414} of sample/OD_{414} of zero palytoxin control.
b. n.d. = not done.

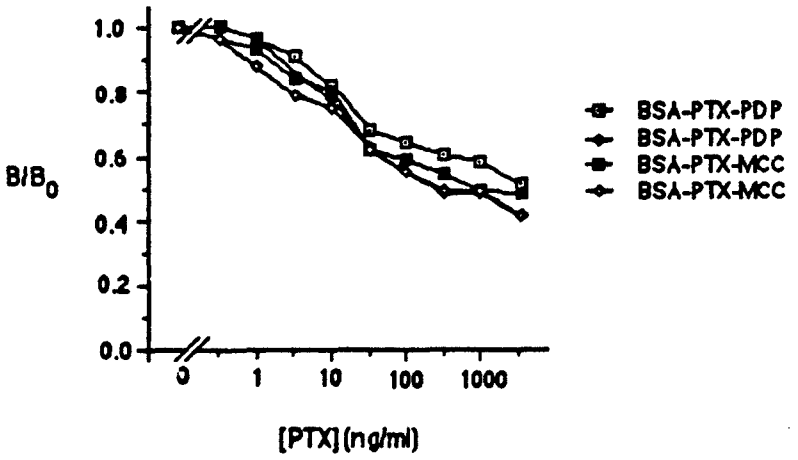


FIGURE 6. Comparison of Coating Antigens in Rabbit Polyclonal Antibody Indirect CIEIA. BSA-PTX-PDP and BSA-PTX-MCC were used to coat ELISA plate wells. The protocol given in Appendix N was followed. Key to symbols: squares = rabbit HBG 007, bleed 4, diluted 1/8000; diamonds = rabbit HBG 007, bleed 4, diluted 1/16000.

The rabbit polyclonal anti thus provided a prototype immunoassay for detecting and quantitating PTX. However, the usefulness of this assay system was limited by its insensitivity (10 µg/ml PTX required for 100% inhibition) and the limited supply of standard antiserum (R7B7). Production of suitable monoclonal antibodies (MAbs) against PTX, and development of a MAb-based immunoassay system was clearly required to overcome these limitations.

Although the rabbit polyclonal antibody indirect CIEIA was not sufficiently sensitive for the requirements of USAMRIID, later observations demonstrated the usefulness of R7B7 when used in conjunction with a MAb in a sandwich ELISA system. Since only 20 ml of our stock of R7B7 remained by the beginning of Year 4 of this project and our contract with the U.S. Army required us to supply them with reagents for immunoassays developed, we wished to maintain a large stock of this "standard" reagent. Hyperimmunization of three new rabbits was therefore started during early May of 1990, with KLH-PTX-MCC. After five injections of KLH-PTX-MCC, all three rabbits exhibited end point titers in the indirect ELISA on microtiter plate wells coated with BSA-PTX-MCC of 1 in 1 x 10^6 to 1 in 4 x 10^6. All three rabbits were therefore sacrificed and exsanguinated, yielding more than 300 mls of serum with an ELISA titer greater than 1 in 16 million (1 in 1.6 x 10^7).

Comparison of the performance of these new hyperimmune rabbit antiserum pools with R7B7 in indirect CIEIA (see later section on development of rabbit polyclonal antibody indirect CIEIA), was initiated in October, 1990. Data indicated that though binding of R7B7 serum to solid phase BSA-PTX-MCC was inhibited by free PTX, binding of serum from the more recently hyperimmunized rabbits was

not. After some consideration, we determined the reason for this phenomenon. Rabbit #7 was hyperimmunized with KLH-PTX-PDP, but more recently hyperimmunized rabbits were immunized with KLH-PTX-MCC. Thus, serum from these three rabbits would contain antibody against the SMCC linker present in both KLH-PTX-MCC and BSA-PTX-MCC conjugates. Binding of this anti-MCC linker antibody to BSA-PTX-MCC in the CIEIA would not be inhibited by free PTX. We felt that this phenomenon could be overcome by substituting PTX hapten conjugated to BSA via a different linker in place of the BSA-PTX-MCC coating antigen.

Due to the observed instability of BSA-PTX-PDP, PTX was directly (no linker) conjugated to BSA via the EDC method [38]. BSA-PTX conjugate prepared using this method was a satisfactory coating antigen with sera from the more recently hyperimmunized rabbits in both the ELISA and the CIEIA systems. Though the PTX binding activity of these antisera was more inhibitable by free PTX on wells coated with BSA-PTX than on wells coated with BSA-PTX-MCC, this activity was still not fully inhibitable (Figure 7).

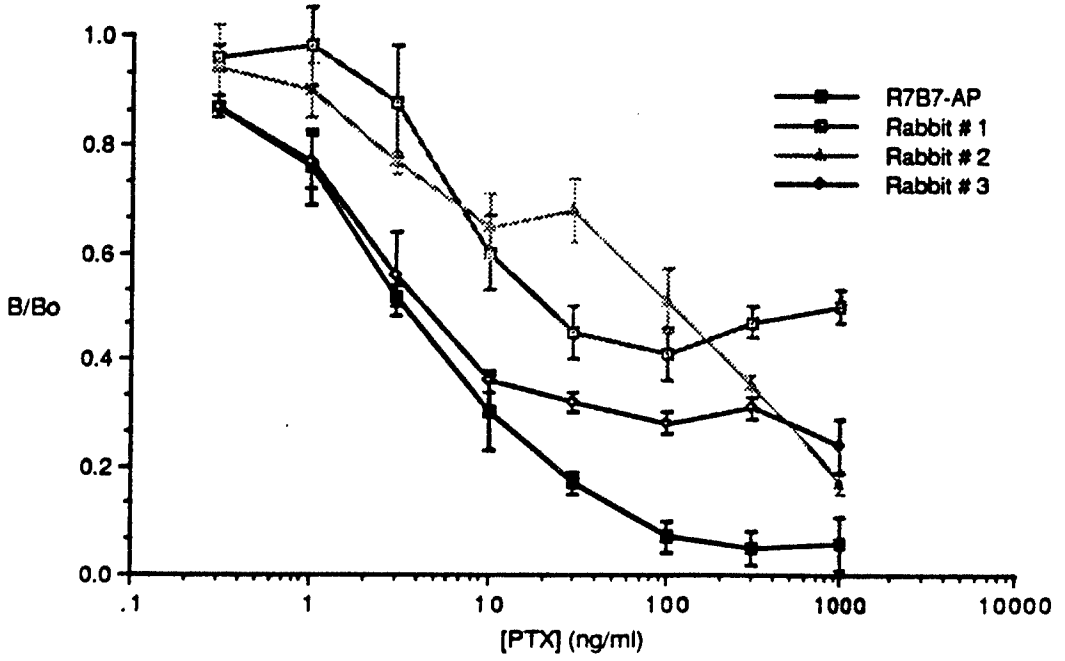


FIGURE 7: Comparison between the Performance of the New Anti-PTX Rabbit Antisera and R7B7 in the CIEIA System

DEVELOPMENT OF MOUSE MONOCLONAL ANTIBODY BASED IMMUNOASSAYS

Production and Characterization of Mouse Monoclonal Antibodies.

Mice were immunized with several different palytoxin conjugates at various times during the four years of this project with a view to producing hybridomas. During Year 1, mice were

hyperimmunized with KLH-PTX-MCC and three fusions were carried out. Mouse monoclonal antibodies (MAbs) to palytoxin were selected for further study using the following criteria: firstly the intensity of their ELISA reactivity with BSA-PTX-PDP coating antigen (see Appendix J), and secondly the concentration of free palytoxin necessary to inhibit the MAb's reactivity with BSA-PTX-PDP (see Appendix N). The latter criterion was intended to select MAbs that would provide a CIEIA with the highest possible sensitivity for palytoxin detection.

The results of three fusions performed durin Year 1 for hybridoma production are summarized in Table 5. Fifty-nine primary hybridomas were shown by the primary ELISA screen to be secreting antibody against palytoxin. Selection by the second criterion described above demonstrated that the BSA-PTX-PDP binding activity of antibody species from at least four of these hybridomas was specifically inhibitible by free palytoxin. Cloning selected primary hybridomas by limiting dilution produced several stable monoclonal hybridoma lines, of which two, designated 73D3 and 89F9, were selected for further studies.

TABLE 5. Summary of Hybridoma Production During Year 1

Date	Fusion Number	# of Cultures Plated	# of Positives in 1° Screen	# with Useful Antibodies	Stable Clones Produced
3/2/87	1	258	9	0	NONE
6/1/87	2	265	38	3	73D3
8/14/87	3	465	12	1	89F9

The isotypes of 73D3 and 89F9 were determined. Both proved to have IgG_1 heavy chains and kappa light chains. These two MAbs (like certain other MAbs from these Year 1 fusions) exhibited similar inhibition curves with free palytoxin (IC_{50} values, the minimum detectable dose, and the slopes of the inhibition curves were indistinguishable). There was no evidence of an additive effect when the two antibodies species were mixed. Although they are derived from immune cells arising in two different mice, there was no other way to distinguish these antibodies. During Year 1, both 73D3 and 89F9 were successfully grown as immunoglobulin-producing ascites tumors, and 73D3 MAb was purified to at least 95% purity using chromatography methods described by Bio Rad Laboratories (Richmond, CA) with AffiGel Blue followed by Biogel P-100 columns.

During Year 3, additional studies were performed on the MAbs from the stable hybridoma lines produced in Year 1, to investigate further the similarities between them. MAbs were tested in a CIEIA system utilizing optimally diluted alkaline phosphatase (AP)-labelled 73D3 conjugate and microtiter wells coated with BSA-PTX-MCC (see later sections on production of AP-MAb conjugates and development of direct MAb CIEIA), to ascertain whether any of them inhibited the binding of AP-73D3 to PTX. Results obtained showed

a high level of inhibition by all MAbs, confirming that all these MAbs were directed against either the same epitope, or epitopes that are close together, on the PTX molecule.

Prior to Year 4 of this project, all fusions performed for generating anti-PTX MAbs had utilized spleen cells from BALB/c mice hyperimmunized with KLH-PTX-MCC. During Year 4, we investigated whether a less inbred strain of mouse might respond to a wider repertoire of epitopes. Swiss-Webster mice were hyperimmunized with KLH-PTX-MCC until they developed a high antibody titer against PTX (1 in 10^5). Spleen cells from two of these mice were used in a fusion experiment for hybridoma production. This fusion yielded 42 primary hybridomas that reacted with BSA-PTX-MCC in the screening ELISA, of which 7 were inhibited by free PTX in the indirect CIEIA (see Appendix N).

Four PTX reactive subclones were recovered after cloning, and their culture supernatants tested for their ability to inhibit binding of AP-73D3 to BSA-PTX-MCC in the direct CIEIA (see later sections on production of AP-MAb conjugates and development of direct MAb CIEIA). Antibodies inhibiting AP-73D3 in this CIEIA would likely be directed against the same epitope on the PTX molecule as 73D3, or closely related epitopes on the PTX molecule. Antibodies that did not inhibit AP-73D3 in this CIEIA would be more likely directed against other, distinct epitopes, and might therefore sandwich with 73D3 MAb in a sandwich ELISA (see later section on development of a sandwich ELISA). These experiments showed that two of the supernatants did inhibit AP-73D3 binding, while the other two did not. Antibody, purified with Protein A-Sepharose from culture supernatants from the latter two clones, was used to coat microtiter plate wells. A direct sandwich ELISA using these coated plates with AP-73D3 failed to detect free PTX, indicating that in spite of the CIEIA results, neither of the new MAbs actually did sandwich with 73D3 MAb. These antibodies did sandwich with R7B7-AP, however, suggesting that they were functioning as capture antibodies. Because MAbs from these clones reacted with palytoxin, inhibited AP-73D3 in the CIEIA, and sandwiched with rabbit antibody but not with 73D3 MAb in the sandwich ELISA, they were classified as "73D3-like".

Inactivation of Palytoxin Cytotoxicity by 73D3 MAb.

In the background section of of this report, we describe the use of an *in vitro* cytotoxicity assay with EL-4 cells to demonstrate the biological activity of palytoxin. The method for this assay is given in Appendix S. This assay was employed to determine whether MAb 73D3 had toxin-neutralizing activity. MAb 73D3 was found to inactivate the *in vitro* cytotoxicity of palytoxin for EL-4 cells in this system. As shown in Figure 8, five µg of antibody was capable of inactivating 85 to 98% of the cytotoxicity resulting from 25 pg/ml palytoxin (a LD_{99} dose). The inactivation was slightly more effective when palytoxin and antibody were preincubated 15 minutes or longer prior to the addition of EL-4 cells.

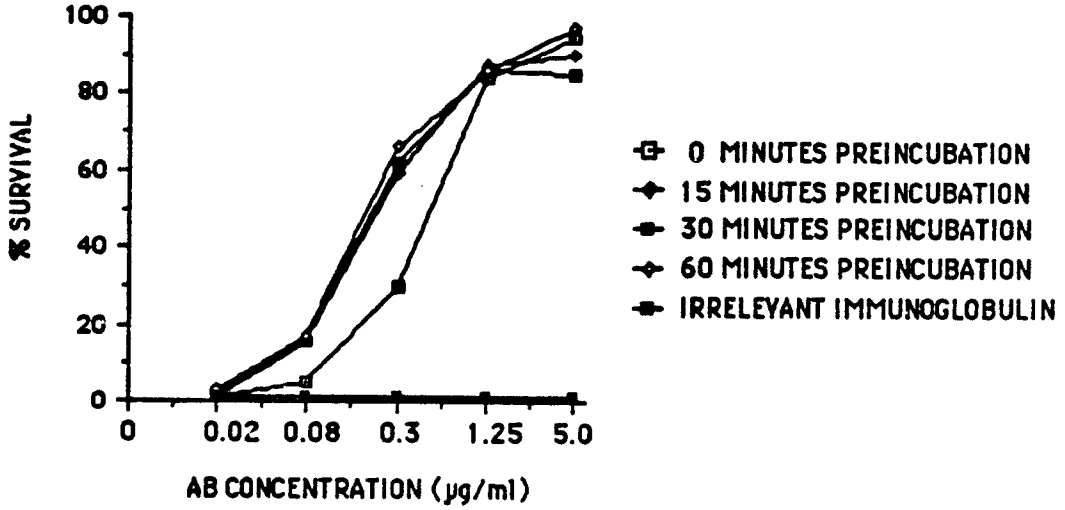


FIGURE 8. Monoclonal Antibody Neutralization of Palytoxin in vitro Cytotoxicity. Palytoxin at a previously determined LD_{99} (25 pg/ml) was preincubated with 5 µg anti-palytoxin 73D3 for various periods of time. 10^5 EL-4 cells were then added to replicate culture wells. After 18 hours under standard culture conditions, 0.1 µCi ^{14}C-L-leucine was added and the cultures incubated an additional two hours before harvesting and processing to determine uptake of radioactive label. 100% survival values were determined from a series of cultures containing culture medium and 5 µg/ml of irrelevant imunoglobulin.

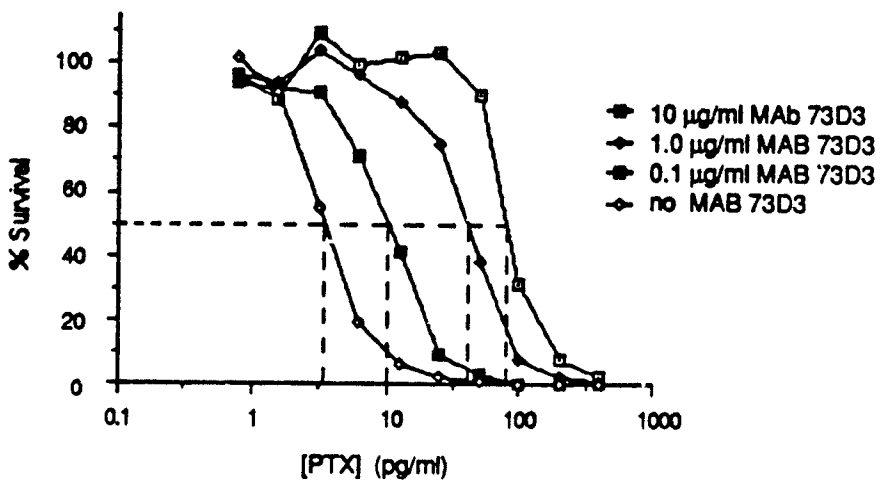


FIGURE 9. Palytoxin Cytotoxicity Curves in the Presence of Anti-Palytoxin Antibody. 10^5 EL-4 cells were dispensed to microwell cultures, followed by monoclonal antibody 73D3 and palytoxin at the indicated concentrations. Incubation and processing to determine cell survival was as described for Figure 8. Dashed lines indicate graphical interpolation of IC_{50} values.

Increasing amounts of antibody had the ability to neutralize increasing amounts of palytoxin, as shown in Figure 9 and Table 6. Figure 9 is based on means obtained in the first three experiments conducted. Table 6 includes data from a total of five experiments of this type. From Figure 9, the LD_{50} values for palytoxin in the presence of 73D3 antibody at concentrations of 0, 0.1, 1, and 10 µg/ml are 3, 10, 50, and 90 pg/ml, respectively.

TABLE 6. Effect of Murine Monoclonal Antibody on Palytoxin Cytotoxicity *in vitro*, No Preincubation.

Palytoxin (pg/ml)	No Antibody	MOPC 21 IgG1 (10 µg/ml)	Protein G Purified (10 µg/ml)	73D3.2.1 (1 µg/ml)	From Ascites (0.1 µg/ml)
400	N.D.	N.D.	1.8 ± 1.3	1.0 ± 1.8	N.D.
200	N.D.	N.D.	7.0 ± 5.6	2.1 ± 1.4	N.D.
100	N.D.	N.D.	26.0 ± 21.9	6.5 ± 6.5	N.D.
50	0.9 ± 0.7	1.0 ± 0.1	71.0 ± 30.2	34.1 ± 20.2	4.1 ± 4.4
25	1.7 ± 1.4	1.2 ± 0.7	90.8 ± 15.9	66.2 ± 17.4	13.4 ± 13.3
12.5	5.9 ± 5.2	3.4 ± 4.0	96.3 ± 7.6	82.7 ± 10.2	40.4 ± 18.2
6.25	19.4 ± 16.1	22.1 ± 27.6	95.0 ± 4.1	92.0 ± 7.2	70.9 ± 11.0
3.13	52.6 ± 27.4	47.7 ± 37.1	104.0 ± 9.4	98.1 ± 8.2	88.1 ± 6.0
1.56	83.0 ± 14.8	77.7 ± 31.3	94.7 ± 5.5	92.8 ± 3.6	90.2 ± 3.7
0.78	91.6 ± 13.2	87.3 ± 11.7	99.0 ± 3.0	89.5 ± 6.9	89.8 ± 11.7
0	100	106 ± 1.0	98.8 ± 6.1		

Data are expressed as percent survival ± Standard Deviation

Number of Triplicate Experiments per Data Point (n):

No antibody: n= 5

73D3.2.1 Anti-palytoxin:

10 µg/ml antibody, 400, 200, 100, 1.56, 0.78pg/ml Palytoxin: n = 3

10 µg/ml antibody, 50, 25, 12.5, 6.25, 3.13 pg/ml Palytoxin: n = 4

1 µg/ml antibody, 400, 200, 100 pg/ml Palytoxin: n = 4

1 µg/ml antibody, 50 ---> 0 pg/ml Palytoxin: n = 5

0.1 µg/ml antibody, all Palytoxin doses: n = 5

MOPC 21 IgG1 control (10 µg/ml), all Palytoxin doses: n = 3

In addition to neutralizing some of the *in vitro* cytotoxic effects of palytoxin, monoclonal antibody 73D3 has been shown effective in prolonging the survival time of mice given a LD_{99} dose of palytoxin, as demonstrated in a preliminary study in collaboration with Dr. John Hewetson of USAMRIID [39]. The abstract of this preliminary investigation of these effects is included as Appendix T.

Further male BALB/c mice were primed with pristane and injected intraperitoneally with 73D3 hybridoma cells. A total of 57 ml of ascitic fluid was collected from them. This fluid had an ELISA titer greater than 1 in 300,000. MAb was affinity purified from this ascitic fluid, using Protein A-Sepharose, for preparation of a new batch of AP-73D3 conjugate.

Development of Mouse MAb Indirect CIEIA for Palytoxin.

During the latter part of Year 1 of this project, initial attempts were made to develop a MAb-based indirect CIEIA for palytoxin, using anti-palytoxin MAb 73D3. Indirect CIEIA systems are less cost-effective than direct CIEIAs that utilize enzyme-labelled MAb conjugates, as they require an additional reagent, an additional incubation, and an additional washing step. However, at this time the technology for preparing enzyme-labelled MAb conjugates was not available 'in house' at HBG.

The protocol for the 73D3 MAb-based indirect CIEIA is given in Appendix N, and a representative standard curve is shown in Figure 10. Inhibition ranges from virtually 100% to zero in a palytoxin concentration ranged of 1 to 100 ng/ml. The palytoxin inhibitor concentration resulting in 50% inhibition (i.e. the detection limit for PTX at IC_{50}) in this system was 6.5 ng/ml.

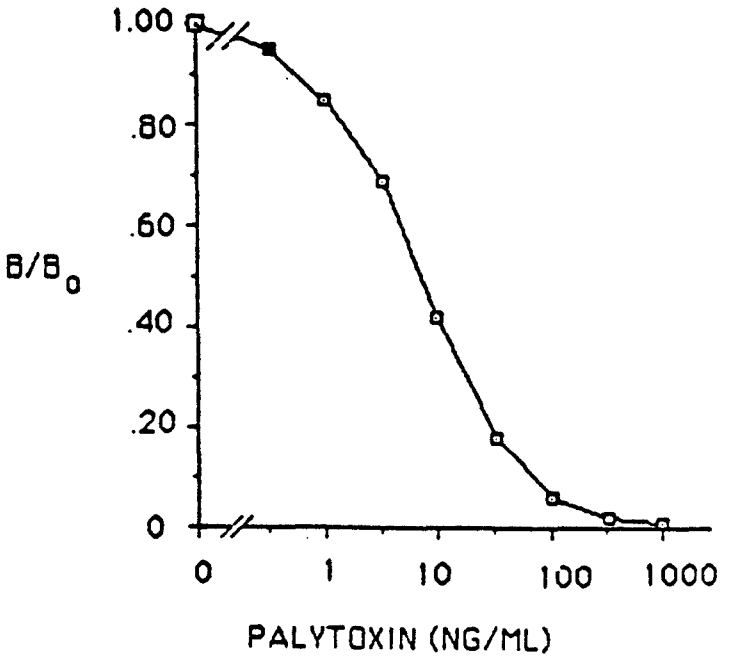


FIGURE 10. Titration of Palytoxin in MAb Indirect CIEIA. The protocol given in Appendix N was followed. MAb 73D3 culture medium supernatant diluted 1/64 was used. Palytoxin was diluted as indicated. B/B_0 values were calculated from OD_{414} readings taken one hour after substrate addition.

The minimum detectable dose (MDD) in this assay is the lowest concentration of palytoxin which had B/Bo values significantly different from the zero palytoxin controls. By Student's unpaired t test, the MDD is 1 ng/ml with a p value of $.025 < p \leq .05$.

Assay Reproducibility:

The reproducibility of the MAb-based indirect CIEIA was assessed by measuring the constancy of IC_{50} values obtained over a period of time. The results of 30 experiments conducted by two different investigators over a period of 72 days were plotted as a scattergram in Figure 11. The mean and upper and lower 95% confidence limits are shown. One experiment fell outside of the upper confidence limit by a great margin and another fell slightly above the upper confidence limit. By the criterion generated from the 95% confidence limits, results obtained on either of those days should be discarded.

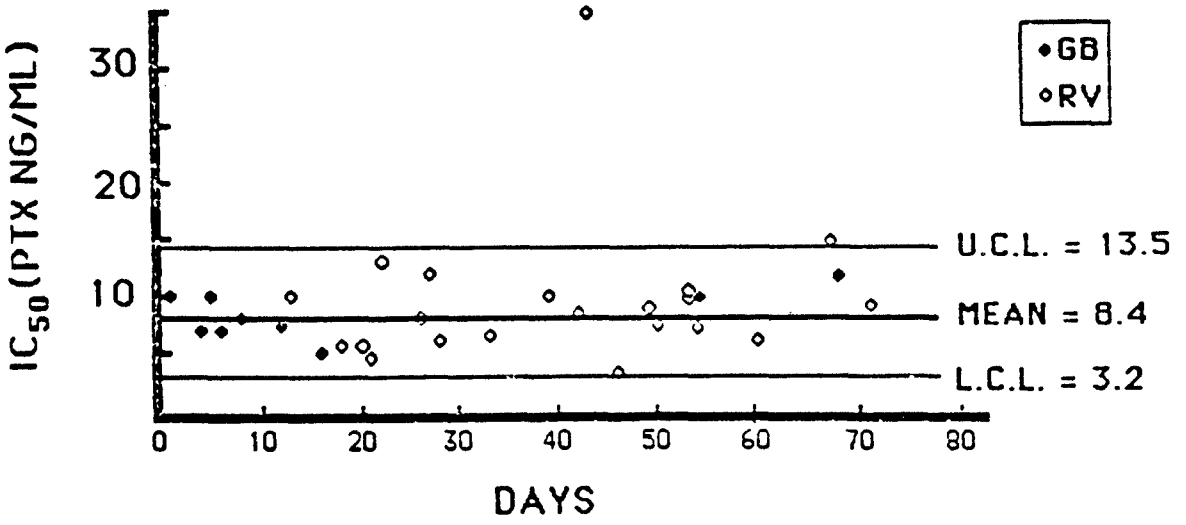


FIGURE 11. Scattergram of IC_{50} Values. IC_{50} values from 30 runs of the MAb Indirect CIEIA are plotted as a function of time. MAb 73D3 was used according the protocol given in Appendix N. GB and RV are two investigators in our laboratory.

Specificity of MAb Indirect CIEIA and Comparison with *In vitro* Cytotoxicity Assay

Exposure of palytoxin to various chemical reagents has been reported to cause decomposition and loss of biological activity [5]. We therefore tested the effect of these reagents on the immunogenicity of palytoxin in our indirect CIEIA. Two *N*-acylated palytoxin derivatives, three other natural marine toxins, ouabain (a known inhibitor of palytoxin binding to Na, K-ATPase [40]), and five other small, polyoxygenated natural products which bear some superficial structural similarities (but no other known natural association) to palytoxin were compared with regard to their ability to inhibit antibody binding in the MAb indirect CIEIA (Table 7). *N*-pyridyldithiopropionylpalytoxin and palytoxin treated with 1N HCl or 1N NaOH were serologically as reactive as native palytoxin. *N*-acetylpalytoxin (*N*-AcPTX), heat-treated

palytoxin and Clorox-treated palytoxin were approximately 10-fold less reactive than native palytoxin. Of the other compounds tested, only amphotericin B and filipin showed weak reactivity.

TABLE 7. Palytoxin Immunoassay Specificity

Compound	Minimum Detectable Dose	IC_{50} (ng/ml)
Palytoxin	1 ng/ml	8.9 ± 4.9
PTX-PDP[1]	2 ng/ml	13.0
N-Acetylpalytoxin	5 ng/ml	30.0
Heated-treated palytoxin[2]	30 ng/ml	81.0
1 N HCl/palytoxin[3]	3 ng/ml	8.7
1 N NaOH/palytoxin[4]	3 ng.ml	4.8
10% Clorox/palytoxin[5]	10 ng/ml	56.0
Okadaic acid	>1 µg/ml	n.m.[6]
Tetrodotoxin	>1 µg/ml	n.m.
Lyngbyatoxin A	>1 µg/ml	n.m.
Ouabain	>1 mg/ml	n.m.
Rhamnose	>1 mg/ml	n.m.
Amphotericin B	1 mg/ml	n.m.
Filipin	100 µg/ml	n.m.
Monensin	>10 µg/ml	n.m.
Nystatin	>10 µg/ml	n.m.

1 *N*-pyridyldithiopropionylpalytoxin
2 An aqueous solution of palytoxin maintained at 100 °C for 12 hours.
3 Palytoxin treated with 1N HCl for 1 hour at room temperature.
4 Palytoxin treated with 1N NaOH for 1 hour at room temperature.
5 Palytoxin treated with 10% (v/v) Clorox for 1 hour at room temperature.
6 Not measurable at the highest concentration tested.

In view of the above results on the effect of various physical and chemical agents on the immunological activity of palytoxin, it was decided to measure the effect of these agents on the biological activity of palytoxin. This biological activity was measured by an *in vitro* cytotoxicity assay. The method for this assay is given in Appendix S. The results of these experiments (Table 8) demonstrate that the biological activity of palytoxin was severely impaired by heating, or treatment with hydrochloric acid, sodium hydroxide and Clorox.

TABLE 8. In vitro Cytotoxicity of Untreated and treated Palytoxin

Treatment	IC_{50} (pg/ml)
Untreated palytoxin	5
N-Acetypalytoxin	2,000
Heat/palytoxin	>1,000,000
1 N HCl/palytoxin	>1,000,000
1 N NaOH/palytoxin	500,000
10% Clorox/palytoxin	>1,000,000

These results suggest that these chemical and physical treatments cause decomposition of palytoxin, thus reducing its toxicity. It is interesting to note that immunologically reactive epitope of the molecule survives most of these treatments relatively unscathed.

Correlation between the Indirect CIEIA, *In Vitro* Cytotoxicity Assay, Mouse Bioassay and U.V. Spectroscopy.

Ten coded "mock unknown" samples were diluted in 0.9% NaCl, and tested by mouse bioassay, indirect CIEIA, and the cytotoxicity assay. Groups of five female Swiss Webster mice were weighed, injected intraperitoneally with 0.1 ml of unknown samples coded, "A-J", and then time to death was recorded. Mean weights (kg) and time to death (min) were used to calculate the concentration of the administered dose, by the following equation:

$$\log \text{dose palytoxin } (\mu g/kg) = \frac{\log (\text{time to death (min)}) - 1.694}{(-0.601)}$$

The equation was derived from linear regression of the sample data time points against the "true" value as determined by UV spectroscopy at 263 nm (ε_{263} = 23,600) [5,41]. *In vitro* cytotoxicity assays of unknown samples were performed as described in Appendix S. Indirect CIEIAs of unknown samples were performed as described in Appendix N.

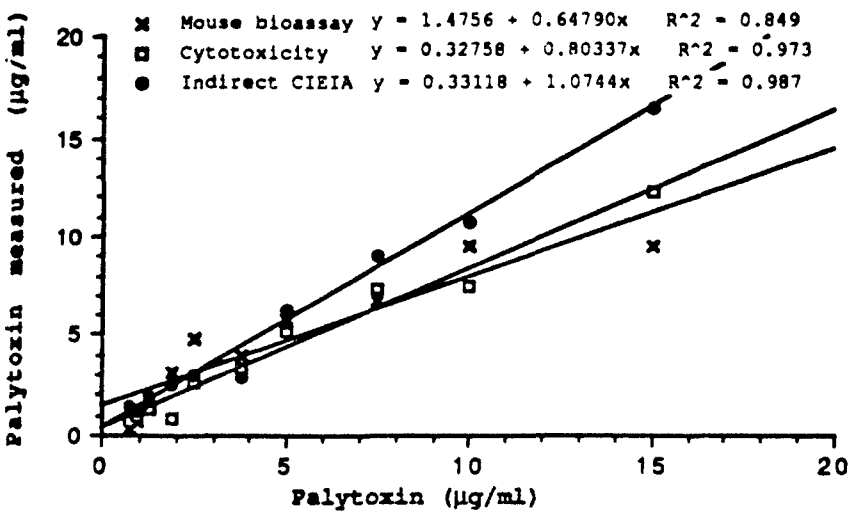


FIGURE 12. Correlation of Indirect CIEIA, IVCTT, and Mouse Bioassay for Palytoxin

The results shown in Figure 12 illustrate that assay of unknown samples by the indirect CIEIA system provided the best correlation with UV spectroscopic determination (slope = 1.07) and

the least variance in the data over the range of measurements (R^2 = 0.987). The mouse bioassay was the least accurate method, as reflected in a reduced recovery of palytoxin from unknown samples (slope = 0.65) and a greater variance in the determined values (R^2 = 0.849). The variance of the determinations by the cytotoxicity assay was low (R^2 = 0.973), however, the recovery of palytoxin was lower than expected (slope = 0.80). Reduction in biological activity of palytoxin is reported to correlate with ablation of the 263 nm chromophore, therefore, each of these assay methods should have been determining fully active palytoxin. The bias observed with the functional assays (mouse bioassay and cytotoxicity assay) could be explained if a fraction of the toxin were biologically inactive, but remained antigenic. This possibility seems unlikely, considering that each method was correlated to palytoxin concentration determined by UV spectroscopy at 263 nm.

Spike and Recovery Experiments and Investigation of "Parallelism" of Data

The accuracy of an assay system with natural samples can be assessed by spiking samples with known amounts of analyte and comparing recovery (as determined by the assay system) with input (31). Extracts were prepared from seven discrete colonies of *P. tuberculosa* collected at Kahe Point, O'ahu in May, 1987. Each extract was spiked with an amount of palytoxin which would increase the palytoxin content of the diluted samples of the extract by 10 ng/ml. The untreated and spiked extracts were then analyzed by the MAb indirect CIEIA, relative to a standard curve generated with purified palytoxin. The resulting estimates of palytoxin concentrations are presented in Table 9. The mean and the standard deviation of the percent recovery indicate the accuracy of the method.

TABLE 9. Palytoxin CIEIA Spike and Recovery Experiment.

Sample	Crude Extract[a]	Spiked Extract[b]	Increment	% Recovery
	(ng/ml palytoxin)[c]			
1	41.8	52.8	11.0	110
2	2.7	13.6	10.9	109
3	30.5	46.4	15.9	159
4	49.0	58.4	9.4	94
5	15.3	23.2	7.9	79
6	1.9	8.6	6.7	67
7	3.5	13.5	10.0	100
			Mean = 102.7 ± 29.3	

[a] Extracts were prepared by extracting one unit (by weight) of *P. tuberculosa* with two units (by volume) of 70% ethanol in water for 24 hours.

[b] Samples were spiked with palytoxin to provide an increment of 10 ng/ml in the palytoxin concentration of the spiked sample.

[c] ng/ml of the diluted sample placed in the well.

As a test for matrix interference, a sample previously estimated to contain approximately 11.5 µg/ml palytoxin was selected. Serial doubling dilutions of the extract ranging from 1/500 to 1/2000 were prepared. Each dilution step was then subdivided into a series of aliquots. The aliquots were spiked with increasing amounts of palytoxin. Two to four replicate experiments were then conducted to determine the total palytoxin content of each tube by the MAb indirect CIEIA. The recovered palytoxin content of the samples placed in the well are shown in Figure 13.

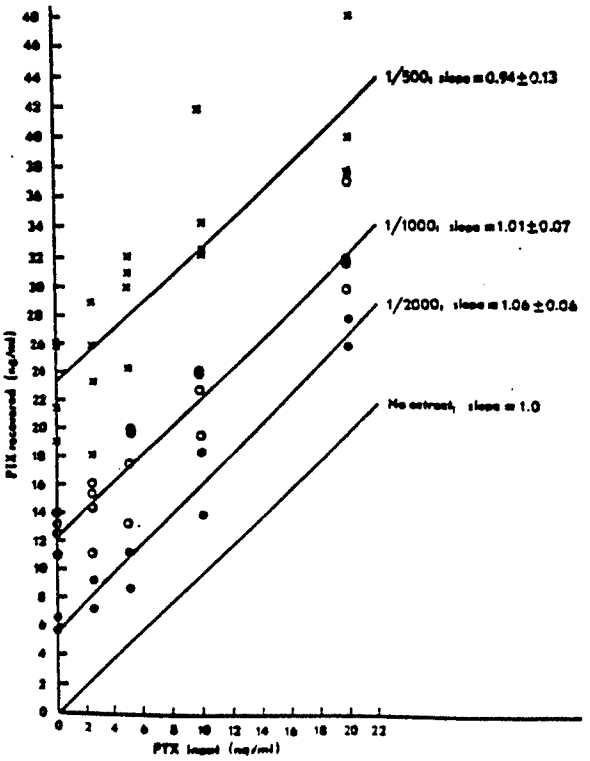


FIGURE 13. Recovery of Palytoxin in Varying Dilutions of a *Palythoa* Extract. Different dilutions of a single *Palythoa* extract were prepared and spiked with varying amounts of palytoxin. Data points from two to four experiments are shown along with calculated regression lines. The protocol presented in Appendix N was followed except that the incubation time for palytoxin and monoclonal anti-palytoxin antibody was four hours instead of the usual one hour.

Subsequently, another batch of extracts was prepared and tested for "parallelism" [42]. The results are shown in Figure 14. Tests run at different concentrations of extract should all give the same calculated value of analyte concentration in the original sample. This would show up in the plot as a horizontal line. Some extracts behaved in the expected manner and some did not.

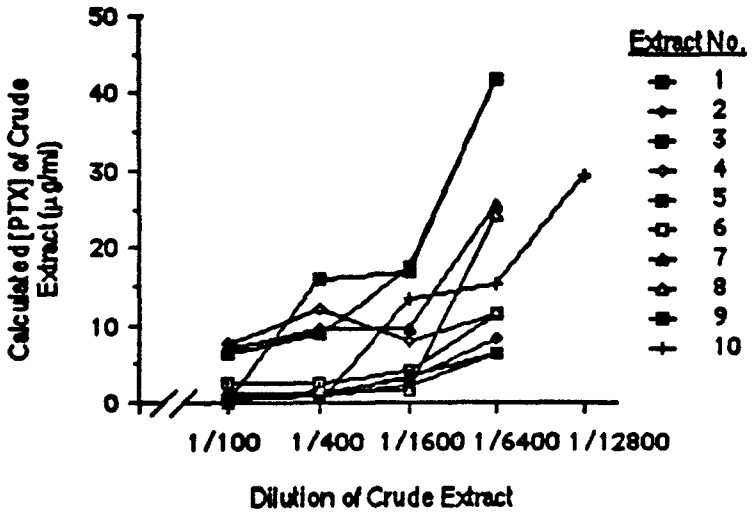


FIGURE 14. Test for Parallelism. Ten extracts were prepared and tested for palytoxin content by the MAb Indirect CIEIA. The calculated value for the palytoxin content of the orignal samples are plotted.

Development of an Indirect "Sandwich" ELISA for Palytoxin.

Because of the "non-parallelism" observed when these crude extracts of *Palythoa* were tested in the MAb indirect CIEIA, the decision was made to investigate development of an indirect sandwich ELISA system for PTX detection, using 73D3 MAb as solid phase "capture antibody", and R7B7 antibody as "detector antibody". An indirect sandwich ELISA system might also enable increased sensitivity, as CIEIAs are less favorable for detecting small amounts of analyte than assays in which the amount of signal increases with increasing analyte concentration.

An indirect sandwich (or immunometric) ELISA was therefore developed. MAb 73D3 was partially purified from mouse ascites fluid by ammonium sulfate precipitation, and used to coat 96 well ELISA plates. Palytoxin was then added, followed by rabbit anti-palytoxin antiserum. Bound rabbit antibodies were detected by addition of goat anti-rabbit immunogobulin alkaline phosphatase conjugate and a suitable alkaline phosphatase substrate. A positive reaction was observed. Each component of the system was then titrated to determine optimal concentrations. The optimized procedure is presented in Appendix O. A typical standard curve showing the effect of increasing palytoxin content on the intensity of color development is presented in Figure 15.

The MDD for this experiment was 300 pg/ml ($.025 < p \leq .05$). The uneven upper portion of the curve is characteristic of 10 replications of this experiment. At this time we do not have an explanation for this irregular dose response relationship at high analyte concentrations. We are confident, however, that the lower portion of the curve is accurately able to measure palytoxin concentrations between 0.3 and 10 ng/ml.

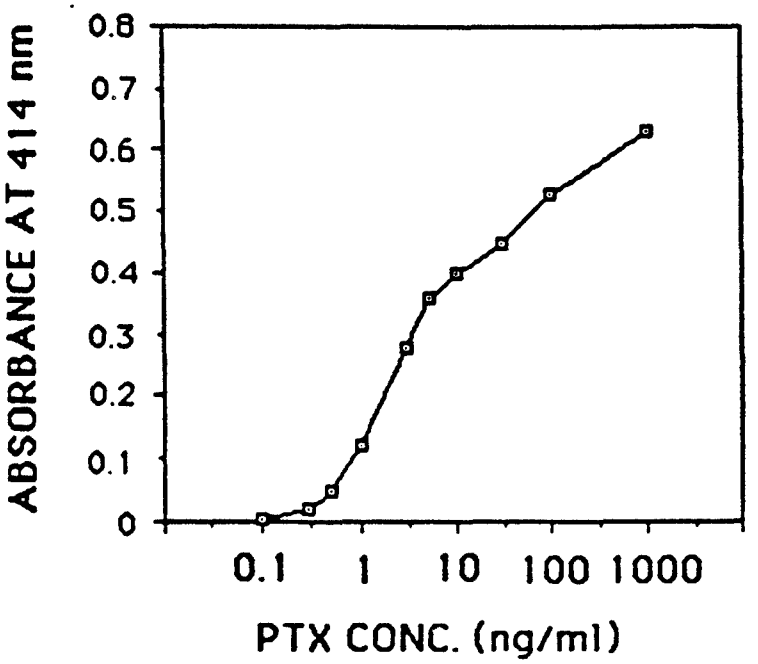


FIGURE 15. Palytoxin Indirect Sandwich ELISA Standard Curve. The procedure given in Appendix O was followed. Absorbance at 414 nm was measured one hour after substrate addition to wells.

Comparison between the Accuracy of the MAb Indirect CIEIA and Indirect Sandwich ELISA with Crude Extracts of *Palythoa*.

Accuracy is the term used to describe how closely unknown determinations agree with known concentrations. A key aspect of accuracy is the ability of an assay to generate the same calculated final concentration of analyte in the starting sample when the samples are tested at various dilutions. Thus, an unknown should contain the same calculated concentration of analyte when tested at a dilution of 1:100 as when tested at a dilution of 1:400. When the calculated analyte concentration is plotted as a function of the dilution factor for each determination, horizontal lines parallel to the ordinate axis should be obtained. Hence, the term "parallelism" is used to describe this test. Ten crude extracts of *Palythoa tuberculosa* were prepared as described in Materials and Methods. Each extract was serially diluted and the palytoxin content determined by the MAb indirect CIEIA. Each extract was tested seven times over a period of a month. The results of these experiments are included as Appendix U. The mean values of each extract dilution which gave results which were interpretable relative to the MAb indirect CIEIA standard curve were plotted in Figure 16 and summarized in Table 10. Most extracts showed significant non-parallelism, as in

the experiment reported in the section earlier in this report describing spike and recovery experiments. In every case the lowest calculated palytoxin content was estimated from a 1/100 dilution of the sample and the highest calculated palytoxin content was calculated from 1/1600 or 1/64000 dilutions of the sample. In the raw data presented in Appendix U, it can be seen that considerable variation among repetitive determinations was observed. This is reflected in the high values for the coefficient of variation (%CV) shown in Table 10. Notwithstanding the high variability of the data and the non-parallel nature of the assay results, repeated testing of the extracts did yield data which could distinguish extracts on the basis of calculated palytoxin content.

Three sets of extracts, consisting of 7, 10, and 10 individual samples were then tested by the MAb indirect CIEIA and the indirect sandwich ELISA. Representative data from the indirect sandwich ELISA are summarized in Figure 17 and Table 11. These data exhibit exactly the same non-parallelism phenomenon observed with the MAb indirect CIEIA.

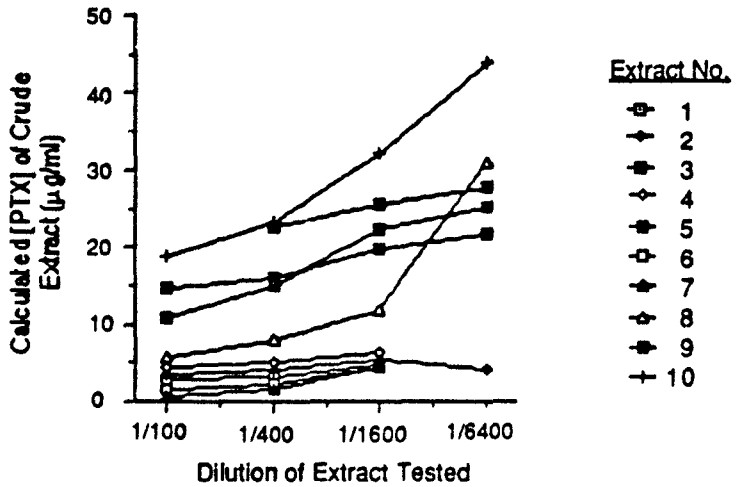


FIGURE 16. Test for Parallelism Using CIEIA to Test Dilutions of Crude *Palythoa* Extracts. Samples from individual colonies of *P. tuberculosa* were soaked overnight in 70% aqueous ethanol to provide crude extracts. These were then subjected to serial dilution as indicated and tested in the Mab indirect CIEIA format. The calculated palytoxin concentration of each sample dilution was determined from a standard curve generated with purified palytoxin. Replicate tests were averaged and the means plotted.

TABLE 10. Summary of CIEIA Parallelism Data

Extract No.	Mean of all Det'ns[1]	LCL[2]	UCL[3]	Range of means of dilutions[4]	%CV[5]
1	3.57	3.25	3.90	2.83 - 4.78	42
2	4.28	3.97	4.59	3.44 - 5.37	33
3	18.32	17.06	19.57	14.70 - 21.63	29
4	6.10	5.24	6.95	4.37 - 6.35	63
5	25.14	23.72	26.56	22.47 - 27.56	26
6	2.92	2.47	3.37	1.53 - 4.48	70
7	2.91	1.97	3.85	0.79 - 4.54	141
8	12.35	10.26	14.44	5.58 - 30.90	81
9	18.36	16.38	20.33	10.70 - 25.17	48
10	29.48	26.66	32.30	18.73 - 44.05	44

1 For each extract, the mean value of all determinations which were interpretable relative to the standard curve were calculated.
2 Mean minus one standard error.
3 Mean plus one standard error.
4 At each dilution tested, mean values were calculated.
5 Percent coefficient of variation of all determinations made on each extract.

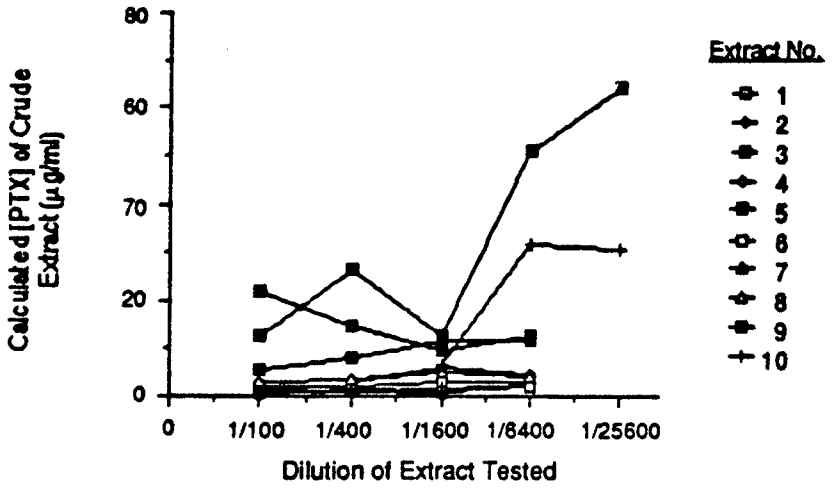


FIGURE 17. Test for Parallelism using Indirect Sandwich Immunoassay to Test Dilutions of Crude Palythoa Extracts. Samples from individual colonies of P. tuberculosa were soaked overnight in 70% aqueous ethanol to provide crude extracts. These were then subjected to serial dilution as indicated and tested in the standard sandwich immunoassay format. The calculated palytoxin concentration of each sample dilution was determined from a standard curve generated with purified palytoxin. Replicate tests were averaged and the means plotted.

TABLE 11. Summary of Indirect Sandwich Immunoassay Parallelism Data.

Extract No.	Mean of all Det'ns[1]	LCL[2]	UCL[3]	Range of means of dilutions[4]	%CV[5]
1	2.57	2.15	2.98	1.93 - 3.01	16
2	1.62	1.28	1.96	1.19 - 2.59	21
3	12.35	8.93	15.77	9.93 - 21.70	28
4	4.80	3.63	5.98	3.31 - 4.42	24
5	42.30	23.37	61.23	12.80 - 64.43	45
6	1.29	1.01	1.57	0.65 - 1.90	22
7	0.82	0.61	1.02	0.70 - 1.01	25
8	4.13	2.91	5.35	3.16 - 4.31	29
9	10.52	6.74	14.31	5.78 - 11.86	36
10	23.32	13.33	33.30	6.66 - 30.70	43

1 For each extract, the mean value of all determinations which were interpretable relative to the standard curve were calculated.
2 Lower 95% confidence limit.
3 Upper 95% confidence limit.
4 At each dilution tested, mean values were calculated.
5 Percent coefficient of variation of all determinations made on each extract.

Investigation of the Effect of Test Buffer on Non-Parallelism Phenomenon.

Several different test buffer milleu were tested in both the MAb indirect CIEIA and the indirect sandwich ELISA systems in an attempt to overcome the non-parallelism phenomenon. Tris-buffered saline (0.01 M Tris, 15 M NaCl, pH7.0) and borate buffers from 0.005 mM to 5.0 mM were tested. We also tried adding Tween 20 (0.01 %), CHAPs (0.01 %), Tergitol NP40 (0.01 %) and antifoam A (0.005% of stock solution from Sigma Chemical Company) to our standard PBS-borate-BSA buffer. None of these variations overcome the non-parallelism phenomenon.

Homogeneous Antibody Sandwich Immunoassay.

One possible explanation suggested for the non-parallelism phenomenon observed in both the MAb indirect CIEIA and the indirect sandwich ELISA systems, was that aggregation of PTX occurred when high concentrations of toxin were present in solution. Thus, when crude extracts of *Palythoa* were titrated from high to low concentration, as in the experiments reported in the previous sections, an apparent increase in PTX concentration might be observed at higher dilutions of sample due to disassociation of aggregates. If a single copy of the 73D3MAb-reactive epitope is present on each PTX molecule (as suggested by data elsewhere in this report), and stable aggregates of PTX molecules were present in samples containing high PTX concentrations, then multiple copies of the epitope reactive with 73D3 MAb should be present in each aggregate, enabling the aggregate to be "sandwiched" between two 73D3 MAb molecules.

An experiment was therefore designed to test this hypothesis. Immunoassay plates were coated with purified monoclonal antibody 73D3 (the "capture antibody"). Dilutions of purified palytoxin were then added followed by biotinylated 73D3 (the "detecting antibody"). Commercial avidin-alkaline phosphatase was then added to demonstrate the presence of bound detecting antibody. As control experiment, rabbit antiserum HBG 007, bleed 6, was substituted for 73D3 trapping antibody. The results presented in Table 12 show that when 73D3 was used as capture antibody, no biotinylated 73D3 detecting antibody was found to be bound to the immunoassay wells. In the control experiment with rabbit capture antibody, biotinylated 73D3 was demonstrated to be bound to palytoxin captured by the capture antibody. These results indicate that PTX molecules do not form stable aggregates when present at high concentration in solution. Further, as "sandwiching" of PTX molecules between two 73D3 MAb molecules was not observed, the results confirm that only one copy of the 73D3 MAb-reactive epitope is present (or accessible to antibody) on each PTX molecule.

TABLE 12. Biotinylated Antibody Sandwich Immunoassays

Capture Antibody	Palytoxin	Detection Antibody	Indicator System	ELISA Result
R7/B7[1]	NONE	BIOT-73D3[2]	AV-AP[3]	0.00[4]
R7/B7	300 ng	BIOT-73D3	AV-AP	0.64
73D3	NONE	BIOT-73D3	AV-AP	0.01
73D3	300 ng	BIOT-73D3	AV-AP	0.00
73D3	300 ng	R7/B7	GARIg-AP[5]	1.00

1 Rabbit HBG 007, Bleed 7.
2 Biotinylated monoclonal antibody 73D3.
3 Avidin-alkaline phosphatase conjugate.
4 Optical density at 314 nm after one hour.
5 Goat anti-rabbit immunoglobulin-alkaline phosphatase conjugate.

At this stage of the project, two indirect enzyme immunoassays had been developed using the available rabbit polyclonal antibodies and mouse monoclonal antibodies. As noted earlier, however, indirect enzyme immunoassay systems are less cost-effective than direct enzyme immunoassay systems as they require an additional reagent, an additional incubation, and an additional washing step. The use of analyte-specific monoclonal or polyclonal antibodies directly conjugated to enzyme overcomes these disadvantages. By avoiding the need for a species-specific enzyme labelled antibody conjugate, the assay cost is reduced in terms of both reagents and time, and in addition, any risk of non-specific reactivity between naturally occuring antibodies in the anti-species conjugate and other components of the system is avoided.

At the beginning of Year 3 of the project, a new P.I. was appointed, who did have experience with technologies for preparing enzyme-labelled polyclonal and monoclonal antibody conjugates. The decision was therefore made to prepare purified antibody immunoglobulin preparations, produce alkaline phosphatase (AP)

labelled antibody conjugates, and develop direct enzyme immunoassay systems.

Purification of anti-palytoxin antibodies

Monoclonal Antibodies: Since it had been the most extensively characterized by indirect enzyme immunoassays and *in vitro* neutralization, 73D3 MAb was selected for further studies. Samples of clarified 73D3 ascitic fluid were applied to a 1 ml column of Protein G-Sepharose (See Materials and Methods section) and Protein A-Sepharose (Appendix K). Protein A-Sepharose columns were eluted with a discontinuous pH gradient (Appendix K) and the protein G columns were eluted either with this same gradient or with 0.5 M ammonium acetate buffer, pH 3 (See Materials and Methods section).

The specific activities in ELISA of ascitic fluid and purified MAb were calculated for each affinity purified matrix. Purification using Protein A-Sepharose and discontinuous pH gradient elution resulted in at least a 10 fold increase in ELISA Specific Activity, with a 15-17% yield of purified protein to total protein in ascitic fluid. This compares to approximately a 6 fold increase in ELISA Specific Activity, with a 10% yield when the same material was affinity purified using Protein G-Sepharose. The Protein A-Sepharose method was therefore used for all subsequent affinity purification of 73D3 MAb from ascitic fluid.

Rabbit R7B7 Antiserum: The precipitate from one bottle of R7B7 ammonium sulfate treated antiserum was washed with 50% ammonium sulfate, redissolved in PBS, then dialysed against several changes of PBS to remove residual ammonium sulfate. The yield of protein from this 10 ml of antiserum was 208.7 mg as 6.2 ml at 33.66 mg/ml.

0.5 ml (16.88 mg) of this material was run into a 1 ml Protein A-Sepharose CL4B column and eluted with citrate-NaCl buffer, pH 3.2 (Appendix K). The yield of eluted protein was 11.0 mg as 10.0 ml of a 1.1 mg/ml solution. When tested in an indirect ELISA system using AP-goat anti-rabbit Ig and BSA-PTX-MCC coated microtiter plate wells, this material had an end point titer greater than 1 in 327,680.

Preparation of Alkaline Phosphatase Conjugates and Preliminary Evaluation in Direct Enzyme Immunoassay Systems.

73D3 Monoclonal Antibody Conjugate: Methods for preparing alkaline phosphatase-MAb conjugates were investigated during Year 3 of this project. Gluteraldehyde methods have not proven to be suitable for MAbs, so an appropriate maleimide conjugation method was sought. Two alkaline phosphatase (AP) conjugates were therefore prepared from Protein A-Sepharose purified 73D3 MAb using the SAMSA/Sulfo-SMCC method [43] (Appendix L). Ratios of Sulfo-SMCC to AP of 25:1 and 5:1 were used with the aim of producing a conjugate with the best activity possible. A large amount of protein precipitation occurred in both preparations during the conjugation step, suggesting that the incubation time stated in the published method was too long. In spite of this

denaturation, both the 25:1 and 5:1 conjugates initially exhibited acceptable levels of activity in ELISAs (reactive at dilutions down to 1 in 8000). The 5:1 conjugate, however, lost 75% of it's activity after 24 hours storage at 4 °C.

In a direct CIEIA system using BSA-PTX-MCC-coated microtiter plate wells (see Appendix P for method), the 25:1 conjugate, at a working dilution of 1 in 1000, reproducibly detected 1-3 ng/ml of palytoxin (IC_{50} of 1.5 - 4.0 ng/ml and IC_{20} of 0.3 - 2.0 ng/ml: see Figure 18). A stability trial was therefore set up on this conjugate (See later section on conjugate stability studies).

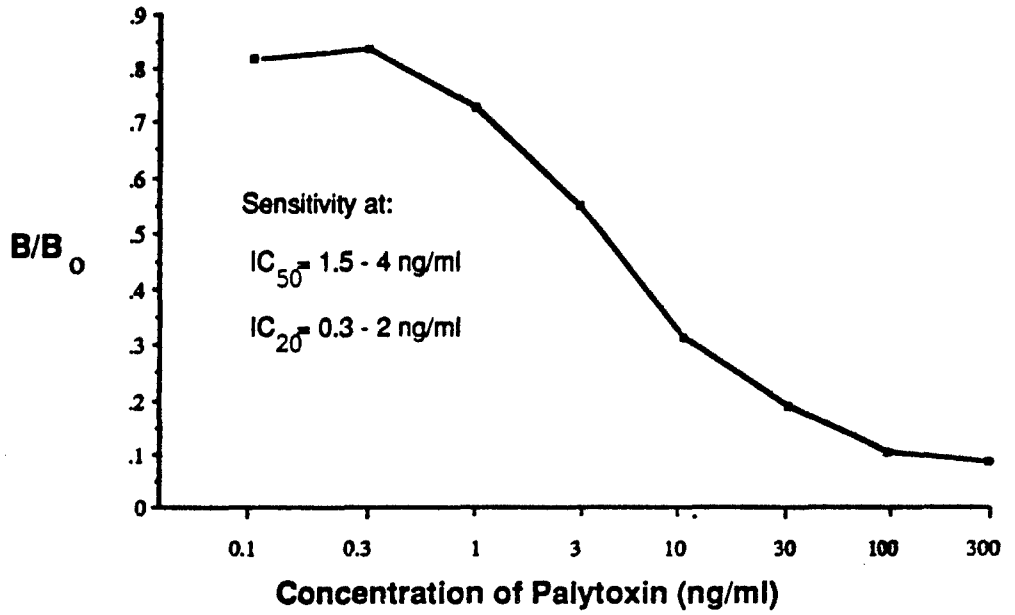


FIGURE 18. Direct CIEIA with AP-73D3: Standard Curve for PTX Detection.

During Year 4 of the project, problems were encountered with conjugating AP to 73D3 MAb using the SAMSA/SMCC method. Two attempts at conjugate preparation were unsuccessful due to "over-thiolation" of immunoglobulin; treatment of 73D3 IgG with SAMSA at a ratio of 100:1 has often resulted in a high ratio of -SH/IgG, and low activity AP- conjugates. Protein A-Sepharose purified 73D3 IgG was therefore treated with SAMSA at a ratio of 25:1. This ratio had successfully produced an active conjugate with another MAb. The first attempt yielded a 3:1 ratio of -SH/IgG, and a low activity AP conjugate. A second attempt using a 25:1 ratio af SAMSA to IgG yielded a 10:1 ratio of -SH/IgG, which produced a high activity AP-73D3 conjugate, with slightly better ELISA activity than the existing AP-73D3 conjugate. Although the original reference for this method [43] recommends an -SH/IgG ratio of 4-7:1, we spent considerable time during the final year of this project attempting to ascertain the optimum ratio of SAMSA/IgG for use with MAb 73D3.

AP-Rabbit (R7B7) Antibody Conjugate: AP conjugates were prepared using Protein A-Sepharcse purified immunoglobulins from rabbit #7, bleed #7 (R7B7), using:

i. the SAMSA/sulfoSMCC method employed for 73D3 (see Appendix L)

ii. the one step gluteraldehyde method of Avrameas [29,30]

iii. SPDP derivatized AP and sulfo-SMCC treated R7B7.

All three conjugates that were initially prepared exhibited unacceptably low activity on BSA-PTX-MCC coated microtiter plates, (reactive only down to a dilution of 1 in 5) and high cross-reactivity on plates coated with BSA alone. Analysis of the SMCC conjugate by a panel of quality control tests indicated that though conjugation had been successful and AP activity was still high, the palytoxin-binding ability of the rabbit antibodies had been drastically reduced.

In view of the poor activity of these AP-conjugates, a horseradish peroxidase (HRPO) labelled R7B7 conjugate was prepared using the sodium metaperiodate method [44]. In a direct ELISA on microtiter plate wells coated with BSA-PTX-MCC using 2.2'-azino-di-(3-ethyl)-benzothiazoline-6-sulfonic acid (ABTS) as chromogen, this conjugate exhibited high activity with an end point titer of 1 in 1600. In spite of our success at preparing this high activity conjugate, it was felt that an HRPO conjugate ELISA system would be less suitable for use with mammalian tissues due to the presence of endogenous peroxidases.

Alternative strategies for preparation of AP-R7B7 conjugates were therefore pursued. A high activity AP-R7B7 was finally prepared during the first quarter of Year 4 of this project, using the dialysis bag modification (Raybould, unpublished observations while employed by Allelix, Inc.) of the one step gluteraldehyde method of Voller et al [45]. This conjugate, at a working dilution of 1 in 200, exhibited excellent performance in a direct sandwich ELISA (see Appendix R for method), with a PTX detection limit of 1-3 ng/ml (Figure 19). A second batch of AP-R7B7 conjugate was successfully prepared during the third quarter of Year 4, using the same method. This second batch of conjugate had a higher working dilution (1 in 600) and gave a lower background in ELISA than the previous batch.

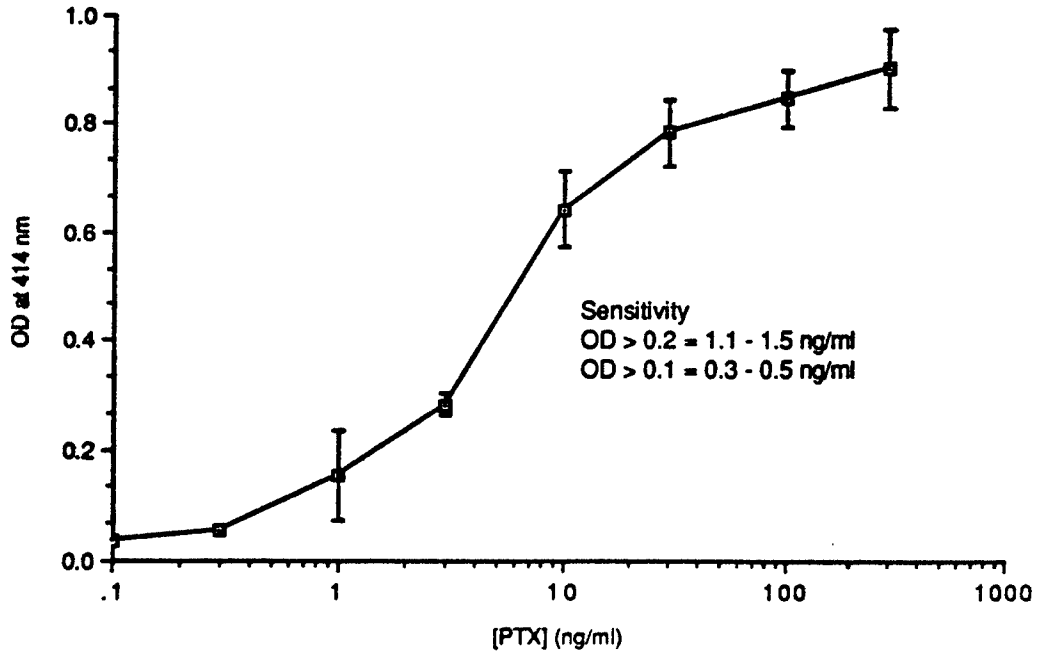


FIGURE 19. Direct Sandwich ELISA with AP-R7B7: Standard Curve for PTX Detection.

AP-PTX Conjugates: A direct labelled AP-PTX conjugate would have applications in several areas. It could be used in a CIEIA system with microtiter plate wells coated with 73D3 MAb, for measuring affinity constants of MAbs, and for development of PTX receptor binding assays that were suggested in another proposal to USAMRDC from Hawaii Biotechnology Group, Inc. After considering several possible synthetic strategies, two different methods for thiolating AP were investigated:

i. AP was thiolated with Trout's reagent (2-iminothiolane) for 1 h. at 22°C with stirring.

ii. AP was derivitized with SPDP, followed by reduction with dithiothreitol (DTT) to produce free thiol groups.

Method i. was unsuccessful. AP thiolated by method ii. was mixed with PTX-MCC hapten at PTX-MCC:AP ratios of 1:1 and 5:1 (Appendix M). AP-PTX-MCC conjugates prepared using both ratios were still bound by 73D3 MAb and exhibited high AP enzyme activity. However, the 5:1 conjugate was usable at a higher dilution than the 1:1 conjugate (1 in 2500 rather than 1 in 500). This conjugate was used to develop a new direct CIEIA system with 73D3 MAb coated microtiter plate wells (see Appendix Q for method). This assay detected palytoxin at an IC_{50} of 10-11 ng/ml and an IC_{20} of 3-4 ng/ml (Figure 20), and had a total test time

similar to the direct CIEIA that utilized AP-73D3 MAb and BSA-PTX-MCC coated microtiter plate wells.

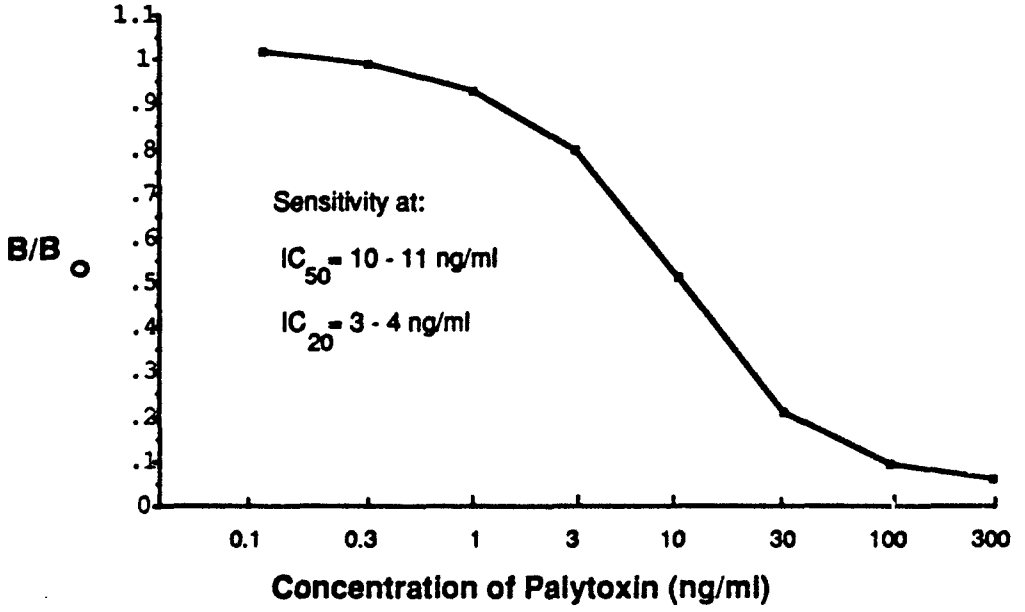


FIGURE 20. Direct CIEIA with AP-PTX: Standard Curve for PTX Detection.

Standard curves for each of the direct enzyme immunoassay systems described above were constructed using standard preparations of PTX dilutions. The standard curves for each system are shown in Figures 18, 19 and 20. The standard curves for the indirect sandwich ELISA and indirect CIEIA systems are shown for comparison in Figures 21 and 22. The sensitivities of the CIEIA systems were calculated from IC_{50} and IC_{20} values. In the indirect sandwich ELISA the concentration of PTX giving an OD_{414} greater than the mean OD_{414} for a series of PTX negative samples plus two standard deviations was employed [46]. In practice, any sample giving an OD_{414} value above 0.1 or 0.2 in this system would be considered positive. The sensitivities for each system, and some of their properties, are compared in Table 13.

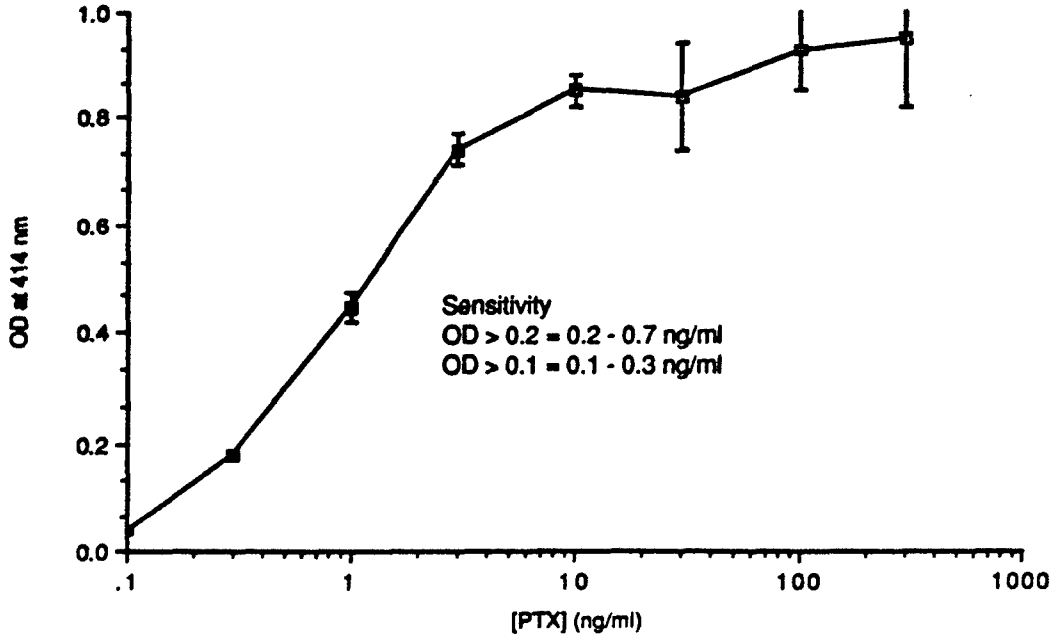


FIGURE 21. Indirect Sandwich ELISA: Standard Curve for PTX Detection.

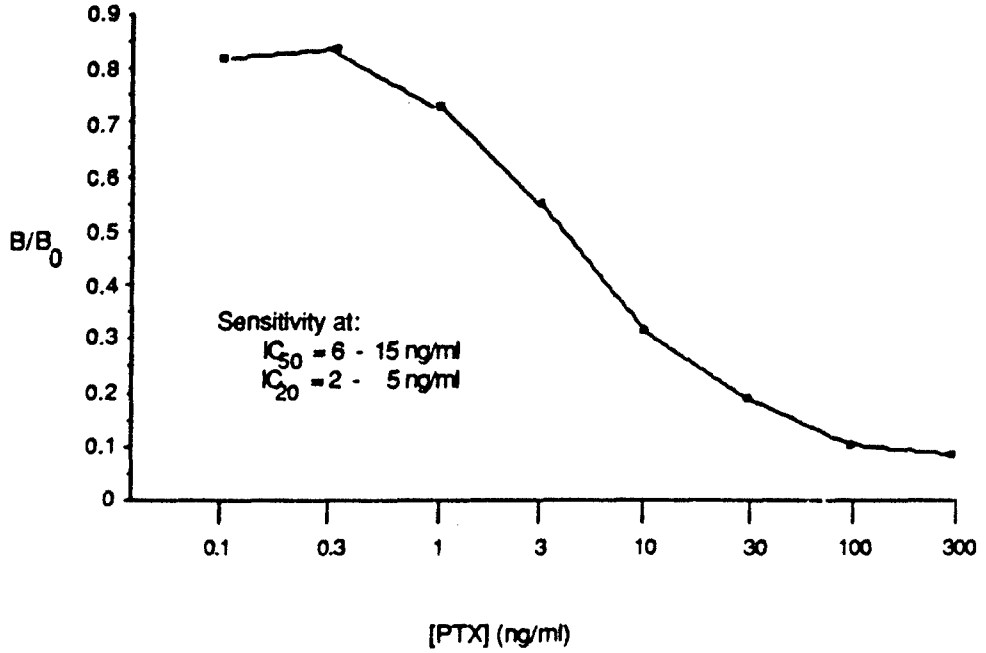


FIGURE 22. Indirect CIEIA with 73D3 MAb: Standard Curve for PTX Detection.

TABLE 13. Comparison of Enzyme Linked Immunoassay Systems for Palytoxin Detection

	Indirect Sandwich ELISA	Indirect CIEIA	Direct CIEIA with AP-73D3 MAb	Direct CIEIA with AP-PTX	Direct Sandwich ELISA
Solid Phase Coated with	73D3MAb	PTX-M-BSA	PTX-M-BSA	73D3 MAb	73D3 MAb
First Reagent Added	Rabbit Anti- PTX	73D3 MAb	AP-73D3 MAb	AP-PTX	AP-Rabbit Anti-PTX
Second Reagent Added	AP-Anti Rabbit IgG	AP-Anti Mouse IgG	-	-	-
Incubation Times	60 min.	60 min.	60 min.	60 min	60 min.
Total Number of Steps (excluding coating, including washing)	7	5	2-3	2-3	2-3
Current Total Assay Time	> 4 hrs.	> 3 hrs.	2 hrs.	2 hrs.	2 hrs.
Sensitivity- minimum conc'n PTX detected (ng/ml)					
@ IC_{50}	0.2 - 0.7[a]	6 - 15	1.5 - 4.0	10 - 11	
@ IC_{20}	0.1 - 0.3[b]	2 - 5	0.3 - 2.0	3 - 4	
Comments	Does not require toxin-hapten = advantage	All require toxin-hapten = disadvantage as this is:		expensive hazardous	Does not require toxin-hapten = Advantage

a OD_{414} > 0.2
b OD_{414} > 0.1

Stability Studies on AP labelled Antibody Conjugates.

AP-R7B7 Conjugate Stability: Liquid AP-R7B7 conjugate in 50% glycerol, the successful preparation of which was described earlier in this Final Report, exhibited no significant drop in activity after 43 weeks (301 days) storage at 0 °C (see Figure 23).

AP-73D3 Conjugate Stability: Several studies on the stability of AP-73D3 conjugates to storage were set up during Year 3 and Year 4 of this project, and the results are summarized in Figures 24, 25 and 26. These figures show the mean optical density value obtained from duplicate tests on the conjugate in the standard ELISA system (Appendix J), using the working dilution established for that conjugate when the stability trial was originally set up. Conjugates that gave optical density readings between 0.2 and 0.8, when tested at their working dilution in the standard ELISA system, were considered satisfactory.

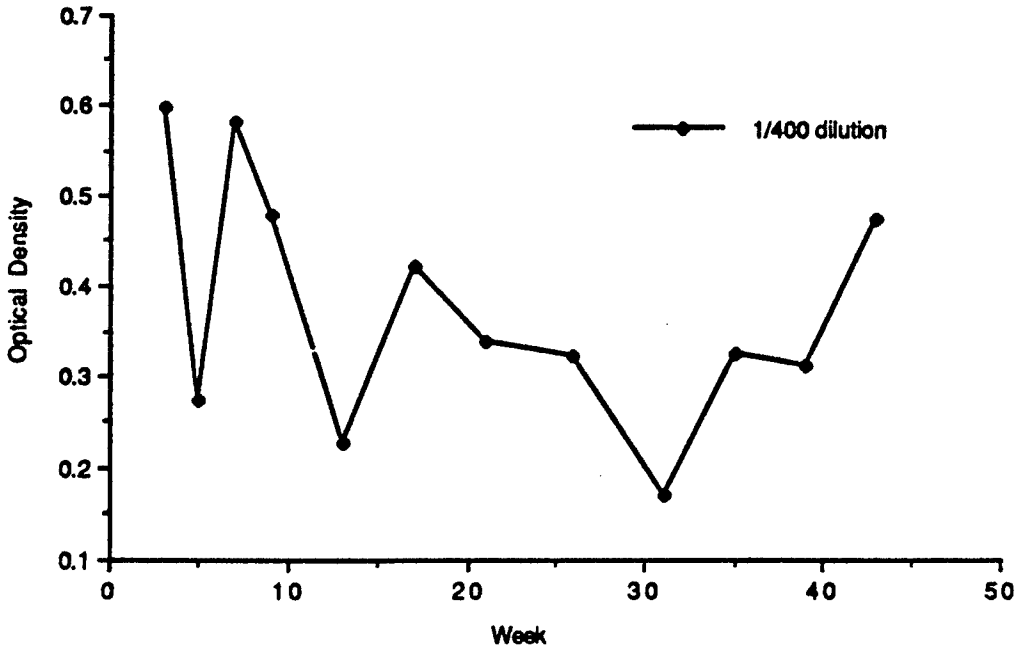


FIGURE 23. Stability of AP-R7B7 Conjugate in 50% Glycerol.

The original liquid AP-73D3 conjugate, (prepared February 9, 1990 and stored in 0.01 M Tris-HCl buffer, pH 6.8 containing 0.1 M NaCl, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 0.5 g/l NaN_3 and 1% BSA) exhibited no significant drop in activity after 50 weeks (350 days) storage at 4 °C (Figure 24).

Another AP-73D3 conjugate was prepared on June 29, 1990, and both liquid and lyophilized aliquots were stored at -20 °C, 4 °C and 37 °C. One set of liquid conjugate aliquots at -20 °C and 37 °C contained 50% glycerol as a stabilizing agent. Another set of liquid conjugate aliquots containing no added glycerol, was placed at 4 °C, as a control for the lyophilized samples. The data in Figure 25 show that activity of the liquid conjugate stored at 37 °C dropped steadily, until after 15 weeks it became unacceptable. After this time period, this conjugate failed to perform satisfactorily even when tested at lower dilutions. The liquid conjugate stored at -20 °C exhibited a gradual, small drop in activity, but still performed acceptably after 24 weeks (168 days). Liquid conjugate, stored without glycerol, still exhibited acceptable activity after 12 weeks storage at 4 °C (Figure 26).

Lyophilization of conjugate resulted in a very slight drop in ELISA activity (Figure 26). Lyophilized conjugate, however, exhibited no further significant drop in activity after storage for 12 weeks (84 days) at either 4 °C or 37 °C (Figure 26).

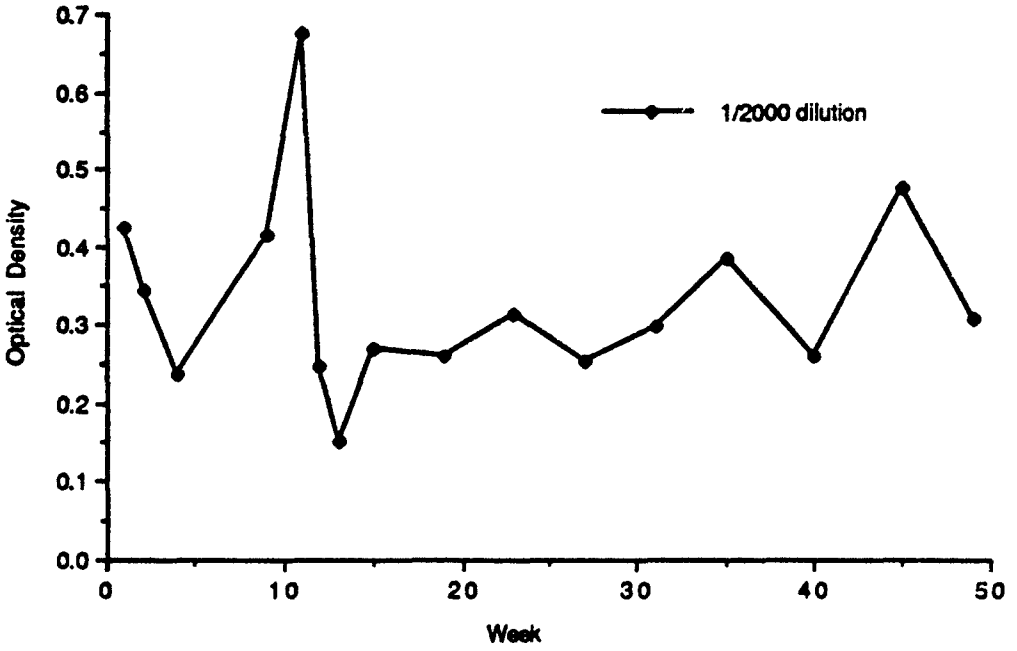


FIGURE 24. Stability of AP-73D3 stored in Tris buffer containing 1% BSA at 4 °C.

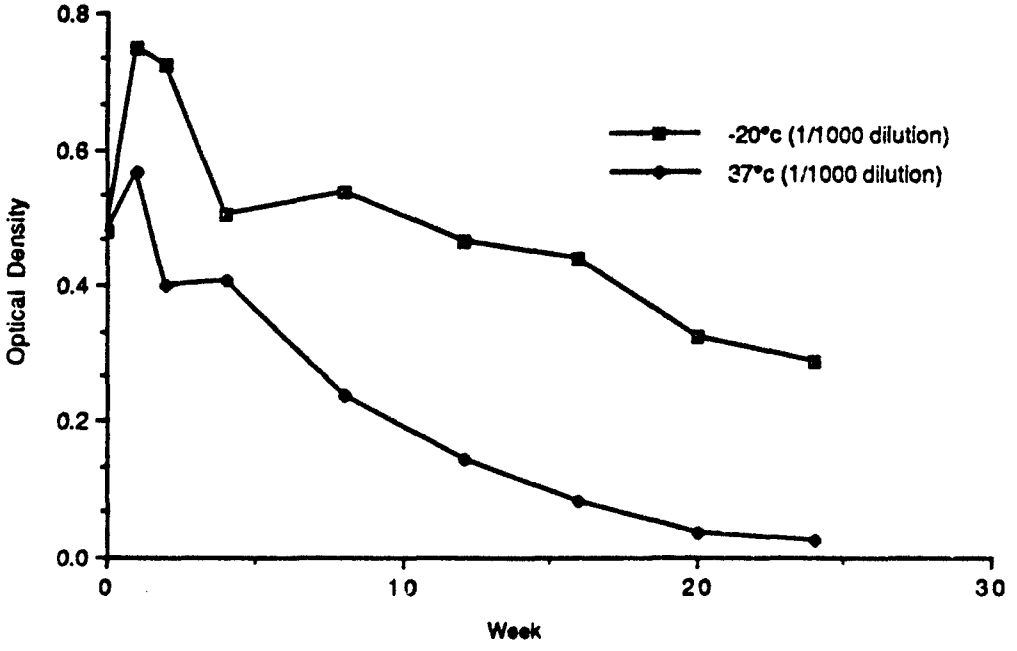


FIGURE 25. Stability of AP-73D3 stored in 50% Glycerol at -20 °C and 37 °C.

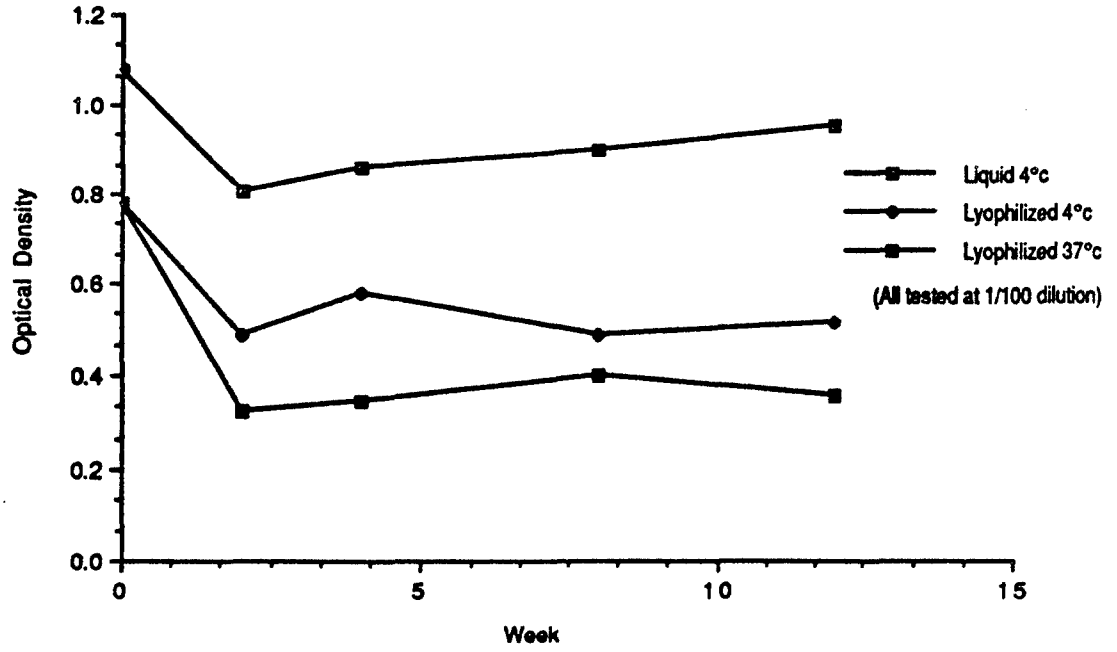


FIGURE 26. Stability of AP-73D3 stored liquid and lyophilized in 1% BSA at 4 °C and 37 °C.

The stability of liquid AP-conjugates stored at 4 °C and lyophilized AP-conjugates stored at 37 °C is significant in demonstrating our ability to produce stable enzyme-antibody conjugate products.

Comparison of CIEIA and Sandwich-ELISA Systems for Detection of Palytoxin in *Palythoa tuberculosa* Extracts

The 70% ethanol extracts (numbered 1-10: 4/6/88) of *Palythoa tuberculosa* from the Okala Island collection of 8/15/87 were initially tested at HBG using the indirect sandwich ELISA and indirect CIEIA systems. Results of these assays are shown in an earlier section of this Final Report. New titrations of nine of the same ten extracts were retested in triplicate in these assays and in the newly developed:

i. Direct Sandwich ELISA with AP-R7B7 and 73D3 coated microtiter plate wells,

ii. Direct CIEIA with AP-73D3 MAb and BSA-PTX-MCC coated microtiter plate wells, and

iii. Direct CIEIA with AP-PTX and 73D3 MAb coated microtiter plate wells.

A mean OD ± standard deviation (SD) was calculated from the triplicate results for each dilution of each sample in each EIA system. In the CIEIA systems, 6 control uninhibited wells were also run in parallel with each sample titration.

A mean B/Bo value was calculated from these data for each sample dilution in each CIEIA system. Concentrations of PTX in each extract were then interpolated from the sample dilutions with mean B/Bo values that fell on the linear portion of each standard curve for that EIA system. In the sandwich ELISA systems, PTX concentrations in each sample were interpolated from the OD_{414} values (mean ± SD) that fell on the linear portion of the standard curve.

Table 14 shows the mean PTX concentrations in each *Palythoa* extract from each of the five EIA systems, together with SD's for each result, and a mean percentage SD for all the samples in each system. With certain exceptions (e.g. extract #3), there was good agreement between the new results obtained for PTX concentrations in each extract from the five EIA systems, and between the new indirect sandwich ELISA and indirect CIEIA results and those obtained with these indirect systems in 1989 (see Tables 10 and 11).

TABLE 14. Determination of Palytoxin Concentration in *Palythoa Tuberculosa* Extracts.

	Palytoxin Concentration (µg/ml) Calculated from Results of:									
Extract No.	Indirect Sandwich ELISA		Direct Sandwich ELISA		Indirect CIEIA		Direct CIEIA with AP-73D3		Direct CIEIA with AP-PTX	
	mean ± S.D.		mean ± S.D.		mean ± S.D.		mean ± S.D.		mean ± S.D.	
1	2.90	1.85	1.53	0.64	3.15	0.22	2.57	0.29	3.00	0
2	0.30	0.30	0.41	0.37	2.46	0.58	1.67	0.63	3.53	0.42
3	3.87	3.43	14.75	0.92	28.36	10.34	6.57	0.99	20.69	1.61
4	2.43	1.07	3.4	0.57	5.71	0.51	2.13	0.76	4.70	0.82
5	11.48	4.43	30.1	0	22.93	3.33	8.97	1.07	22.93	0.92
6	0.86	0.24	0.75	0.01	1.24	0.14	0.88	0.21	1.44	0.06
7	0.43	0.06	0.57	0	0.34	0.05	0.25	0.04	0.21	0.10
9	25.60	16.74	11.3	0.7	18.13	2.57	25.92	9.11	11.00	4.24
10	49.92	19.91	24.9	0	24.27	4.55	22.24	12.52	13.40	0.85
Average %S.D. in mean		53 (± 28)		18 (± 30)		17 (± 9)		27 (± 15)		15 (± 16)

The ten Okala *Palythoa* extracts were also tested in the five EIA systems using sample diluting buffer containing 0.05% Tween 20, to ascertain whether this would reduce variability between results. No significant effect was observed on the sensitivity or standard deviations in any of the EIA systems (data not shown).

In studies performed during Years 1 and 2 of this project, it was noted that the PTX concentrations in the Okala *Palythoa* extracts derived from the indirect sandwich ELISA and indirect CIEIA systems exhibited a significant "non-parallelism" effect. That is, the lowest calculated PTX concentration was given by the lowest extract dilution (1 in 100) and the highest calculated PTX

concentration was given by the highest extract dilution (1 in 1600 or 1 in 6400). A similar analysis was therefore performed on the data from the recent enzyme immunoassays performed on these extracts. Though a wider dilution series was used in the more recent experiments, Figure 27 indicates that while some "non-parallelism" may be present with certain extracts in the indirect sandwich ELISA, it certainly was not observed in the indirect CIEIA or either of the direct CIEIA systems. The low standard deviations previously noted with the indirect CIEIA, and in particular the direct CIEIA using AP-PTX, are again reflected in this figure.

At this point we had successfully developed five enzyme immunoassays for detecting PTX. All of these assays, however, used antibodies produced against PTX haptens derivatized at the amino terminus of the molecule.

The MAb CIEIAs suffered from the disadvantage that they probably detected, in addition to intact PTX molecules, biologically inactive PTX fragments, provided these PTX fragments contained the epitope against which MAb 73D3 was directed. This hypotheseis was supported by the lack of correlation between the results of indirect MAb CIEIAs and <u>in vitro</u> cytotoxicity assays on chemically treated PTX shown in Tables 7 and 8. Development of a sandwich ELISA that utilized antibodies against epitopes on opposite ends of the PTX molecule would overcome this disadvantage, as it should detect only intact palytoxin molecules (i.e. biologically active toxin).

The sandwich ELISAs had the disadvantage that they used rabbit antibodies, of which there was a limited supply. Although further anti-palytoxin polyclonal antibody preparations could be raised in additional rabbits, our data showed that it was difficult to produce a standard polyclonal reagent.

The fact that antibodies in AP-R7B7 bound to PTX molecules after they had been "captured" by solid phase MAb 73D3 in the sandwich ELISA, demonstrated that these haptens stimulate production of antibodies against epitopes on the PTX molecule other than the epitope recognized by 73D3. Unfortunately, we had not succeeded in isolating hybridomas that secreted MAbs with these other specificities. MAbs against epitopes of PTX other than the epitope recognized by 73D3 were therefore still required. Thus, we decided to agressively pursue the synthesis of alternative haptens in which other epitopes on the PTX molecule would be exposed.

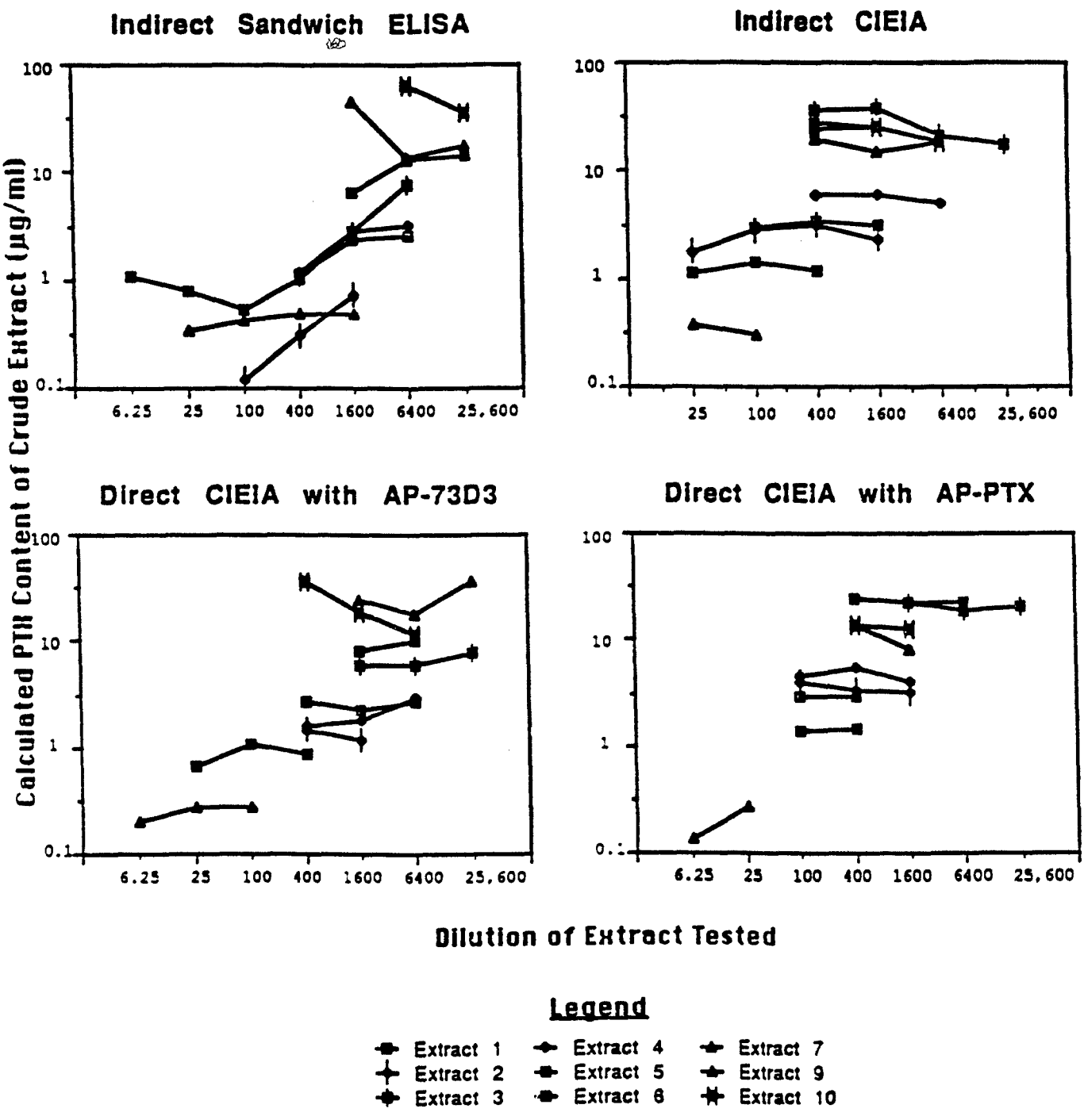


FIGURE 27. Test of Parallelism using Different EIA Systems to Test Dilutions of *Palythoa* Extracts.

Non-amino linked conjugates

One of our objectives was to produce antibodies against different epitopes on PTX. A strategy for accomplishing this was to immunize animals and screen their sera for antibody reactivity with protein conjugates which were linked to palytoxin at positions other than the terminal amino group. To simplify identification of antibodies with desirable specificities for small organic molecules, it is preferable to use a chemically homogeneous hapten for the preparation of the protein conjugates.

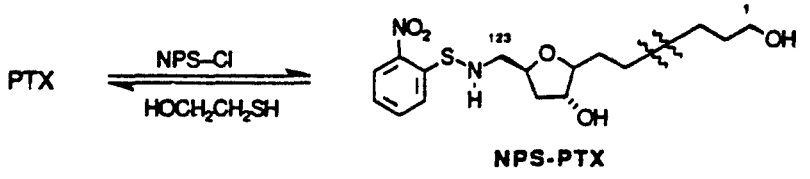
The synthesis of a palytoxin-protein conjugate in which the palytoxin amino group was to be left exposed presented a formidable challenge. There were two major obstacles to be overcome. First, a judicious choice of a protecting group for the palytoxin amino group had to be made. Protection of the amino group during hapten synthesis was necessary because amines are more powerful nucleophiles than are alcohols. Moreover, since the protein-reactive functionality of commonly employed linkers are designed to react with nucleophilic groups such as lysine or sulfhydryl residues, the hapten amino group had to remain protected during the conjugation reaction to prevent polymerization of the hapten and/or cross-linking of palytoxin-protein conjugate molecules. Selective removal of hapten protecting groups from hapten-protein conjugates is complicated by the sensitive nature of proteins and of the hapten-protein linkage, which mandates the use of very mild deprotection conditions. Since hapten-protein conjugates are heterogeneous mixtures of macromolecules with a random attachment of hapten moieties, NMR is not a useful analytical technique for verification of the deprotection step.

The second major obstacle was to develop reaction conditions to enable the selective derivization of one of the 42 hydroxyl groups. To date, the only reported reaction which discriminates among the hydroxyl groups is periodate cleavage of the cis-glycols [47]; obviously a reaction of no value for our objective.

Achievement of our goal was rendered even more difficult by five practical concerns. The limited choice of solvents in which palytoxin is appreciably soluble (H_2O, CH_3OH, DMSO, DMF and pyridine) severely limited the types of reactions which could be employed. The thermal instability of palytoxin also precluded forcing conditions (e.g. elevated temperature). The progress of palytoxin reactions proved difficult tc monitor because minor changes do not have much effect on the molecule's overall polarity and consequently the retention on TLC or HPLC (even the amino acylated derivatives, PTX-PDP and PTX-MCC, are poorly resolved from parent compound). The NMR spectrum is extremely complex and has not yet been fully assigned, thus structural elucidation of reaction products was very difficult. Finally, due of the limited quantities of palytoxin, preliminary reaction conditions had to be tested on < 1 mg of compound. Only if TLC and/or UV results looked encouraging, would the reaction be scaled up for product identification. Because of the high molecular weight (for an organic compound) of palytoxin, it was necessary to use at least

5-10 mg of compound per reaction in order to isolate and attempt to characterize the products. This quantity represented a substantial portion of our yearly toxin production, thus placing severe restrictions on the number of reaction conditions and repetitions which could be studied.

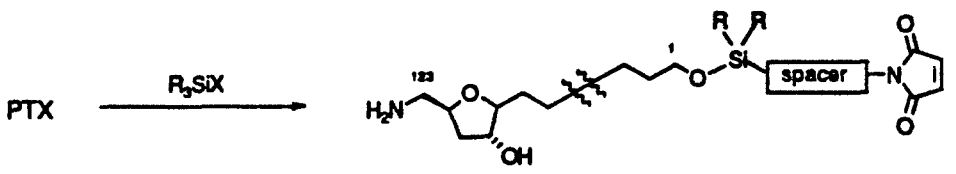
Amino Protection: Our initial synthetic studies used either unprotected PTX or the readily available *N*-acetylpalytoxin (*N*-AcPTX). In the course of our work, Hammock's group at the University of California demonstrated the utility of 2-nitrophenylsulfenyl chloride (NPS-Cl) for the protection of amino groups during hapten synthesis and subsequent protein conjugation [48]. They reported that this protecting group can be removed from the hapten-protein conjugate by brief treatment with β-mercaptoethanol and that all stages of the process could be easily monitored by UV spectroscopy (the NPS group absorbs at 349 nm). This UV absorption also facilitates the measurement of epitope density on the resulting hapten-protein conjugates. After a thorough consideration of possible protecting groups, we decided that the NPS derivative was a logical candidate. After several preliminary TLC-scale experiments with different combinations of solvents and bases, *S*-(2'nitrophenyl)palytoxin sulfenamide (NPS-PTX) was successfully prepared by treatment of PTX with NPS-Cl in pyridine at ambient temperature (Scheme 5; Appendix V). The deprotection method was confirmed by treatment of ~250 μg of NPS-PTX with an excess of β-mercaptoethanol. Regeneration of palytoxin in this reaction was confirmed by TLC and UV.



NPS-PTX

SCHEME 5. Synthesis of NPS-PTX.

Silyl Ether Strategy: Our first approach to the synthesis of a non-amino derivatised hapten sought to take advantage of the different steric environment of the C.1 primary hydroxyl group as compared to the remaining 41 secondary hydroxyl groups. As a model system, we attempted to selectively derivatize palytoxin at this position with sterically demanding trialkylsilylating reagents. The unprotected amino group was expected to consume one equivalent of silyl chloride, however, aminosilanes are known to be unstable to water and we expected the amine to be regenerated upon workup. If selective silylation proved to be possible, we planned to develop a novel heterobifunctional reagent (silylating group on one end; protein-reactive group on the other) from commercially available silanes (Scheme 6).



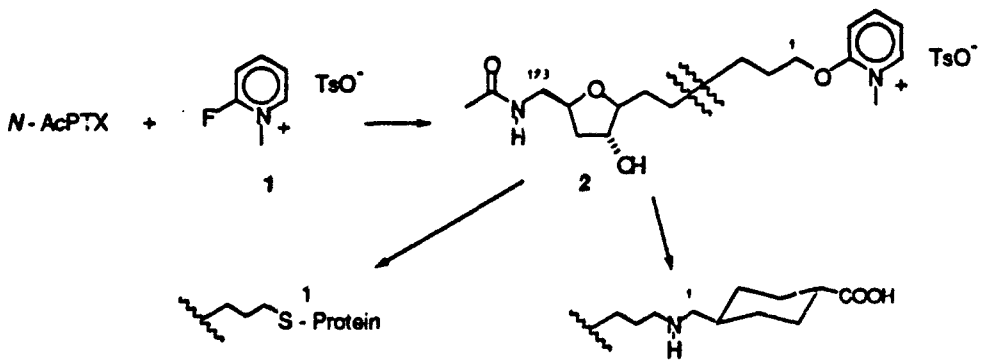
SCHEME 6. Silyl ether strategy.

TLC analysis of our first try to derivatise palytoxin with *tert*-butyl-diphenylsilyl chloride (TBDPSCl) and imidazole (Im) in dimethylformamide (DMF) revealed a new spot which overlapped with PTX. NMR of the crude product looked promising, however, upon purification via ion-exchange, only starting material was recovered. Several additional attempts to derivatise palytoxin with TBDPSCl failed to produce an isolatable silyl ether. Sixty to 70% of the palytoxin was recovered unchanged from these reactions, the remainder of the material was lost either through decomposition or handling. The same result was obtained when the less sterically demanding *tert*-butyldimethylsilyl chloride (TBDMSCl) was employed. Substitution of dimethylaminopyridine (DMAP) or triethylamine (TEA) for Im also failed to effect this transformation. Similar results were obtained when silylation was attempted on *N*-AcPTX. Other attempts were made with the extremely reactive *tert*-butyldimethylsilyl trifluoromethanesulfonate (TBDMS-OTf). The product of this reaction proved to be unreacted PTX contaminated with PTX-derived decomposition products. There was no evidence of silylated PTX-like products.

The NMR spectra of the crude reaction product revealed that there was a huge molar excess of water even though the reaction had been done under anhydrous conditions with dried solvents. NMR analysis proved that this water was present in samples of PTX and *N*-AcPTX even though these samples had been dried in vacuo for several days. We hypothesized that the lack of reactivity with silylating reagents was likely due to hydrolysis of the silylating reagent by this water which is tightly bound to the PTX. Large excesses (20–30 equivalents) of trialkylsilyl chloride were utilized on the hypothesis that the interfering water would be consumed, followed by the desired reaction with PTX. These conditions failed to produce the desired PTX silyl ether.

A sample of Palytoxin was dried by heating at 40 °C for 48 h under vacuum (0.3mm Hg) [49]. We attempted to measure the remaining water by NMR spectra in d_6-DMSO. Qualitatively, the heights of the water peaks in the NMR spectra of this sample and another sample of d_6-DMSO (same volume) from the same bottle are equal. An accurate determination of the remaining water by integration of the water peak was not possible due to the complex nature of the palytoxin spectrum in the region of interest. Attempted silylation of this "anhydrous" palytoxin (TBDMS-OTf/TEA/DMF) was again unsuccessful.

Alkoxy-*N*-alkylpyridinium Strategy: The second approach which we studied entailed the treatment of *N*-AcPTX with 2-fluoro-*N*-methylpyridinium tosylate (**1**). The expected product of this reaction was the 1-(2'-alkoxy-*N*-methylpyridinium)-*N*-acetylpalytoxin salt **2** (Scheme 7). This reagent has been reported to react with alcohols in both anhydrous solvents and aqueous buffers [50]. Since **1** is not sterically demanding, there was no *a priori* reason to predict highly selective formation of one regioisomer. We were hoping that one regioisomer would be preferentially formed due to unpredictable conformational effects. This salt could then be coupled directly with a sulfhydryl group of a carrier protein or reacted with 4-aminomethylcyclohexane carboxylic acid (tranexamic acid) to give a hapten with a carboxylic acid functionality for coupling with an amino residue of the carrier protein.

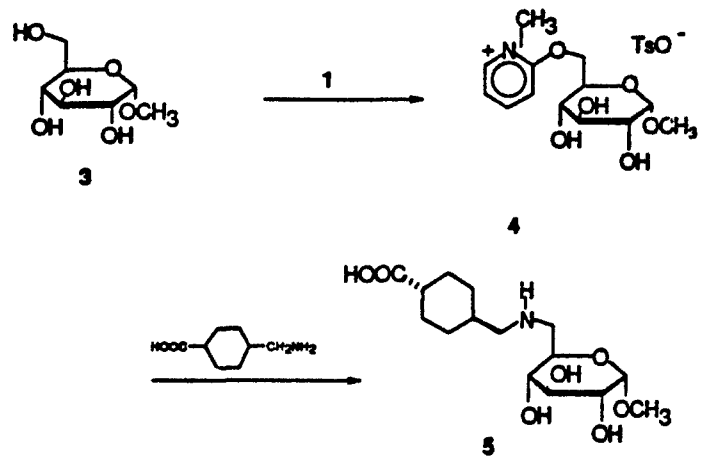


SCHEME 7. Alkoxy-*N*-alkylpyridinium Strategy

We were unable to detect any reaction between *N*-AcPTX and **1** in DMF or phosphate buffer (pH 6.5) under standard conditions reported for these reactions [50]. Approximately 90% of the *N*-AcPTX was recovered in each procedure.

Rather than continuing to consume PTX in failed reactions, we decided to use methyl-α-D-glucopyranoside (**3**) as a model compound to further investigate this reaction. Treatment of **3** with **1** in both dry DMF and in water gave a new very polar product, presumably the salt **4** (Scheme 8). This was not isolated and characterized. Subsequent addition of tranexamic acid gave the expected product **5** in moderate yield (~ 50%). The remaining material was composed of unreacted **3** and several unidentified by-products.

Several variations of this model reaction failed to improve the yield of **5**. Even so, the reaction of **1** with *N*-AcPTX was attempted again in DMF. None of the desired product could be detected by TLC or in the NMR spectrum of the crude product.



SCHEME 8. Alkoxy-*N*-alkylpyridinium Model Study

Palytoxin Carboxylic Acid Strategy: We conceived of our third and most promising strategy for the synthesis of a non-amino derivatised palytoxin hapten upon reading Kishi's recent report [8] of the total synthesis of palytoxin carboxylic acid (PTX-COOH - Figure 28). The isolation of this compound from *P. tuberculosa* was announced by Hirata and Uemura in 1986, however, no details of the isolation or structure elucidation have yet been published. Hirata and Uemura believe it to be a naturally occurring congener although they have not ruled out the possibility that PTX-COOH is an artifact resulting from hydrolysis of the C.9-N.8 amide bond during the isolation of palytoxin [51].

Kishi has reported that, following selective protection of the amino terminus of PTX-COOH with 5-dimethylaminonaphthalene-2-sulfonyl chloride (dansyl chloride), the carboxyl group could be converted to the amide of 1-aminomethylnaphthalene [52]. This result was reported in an interview in *Chemical and Engineering News*, no experimental details or data have yet been published. Based on this information, we reasoned that amide formation between PTX-COOH and the lysine residues of KLH and BSA should be straightforward. Obviously, success of this approach was dependant upon obtaining PTX-COOH either by isolation or selective hydrolysis of the C.9-N.8 amide bond of PTX.

In our efforts to improve the yield of the palytoxin isolation, we monitored the toxin content of the extracts and column fractions by both ELISA and cytotoxicity assays (See Palytoxin Isolation Section for details). We consistantly observed a large loss of immunoreactive and toxic material following the DEAE anion exchange column. Upon learning of Uemura and Hirata's PTX-COOH, we suspected that this decrease in toxicity was due to retention of PTX-COOH on the gel. Therefore, we attempted to recover any acid by washing the DEAE column with a lower pH (3.5) phosphate buffer following normal elution of the PTX. A new UV active fraction was eluted and assayed for toxicity. The cytotoxicity assay would have detected µg quantities of the PTX carboxylic acid if it has activity

comparable to PTX as reported by Uemura. None of the fractions was toxic.

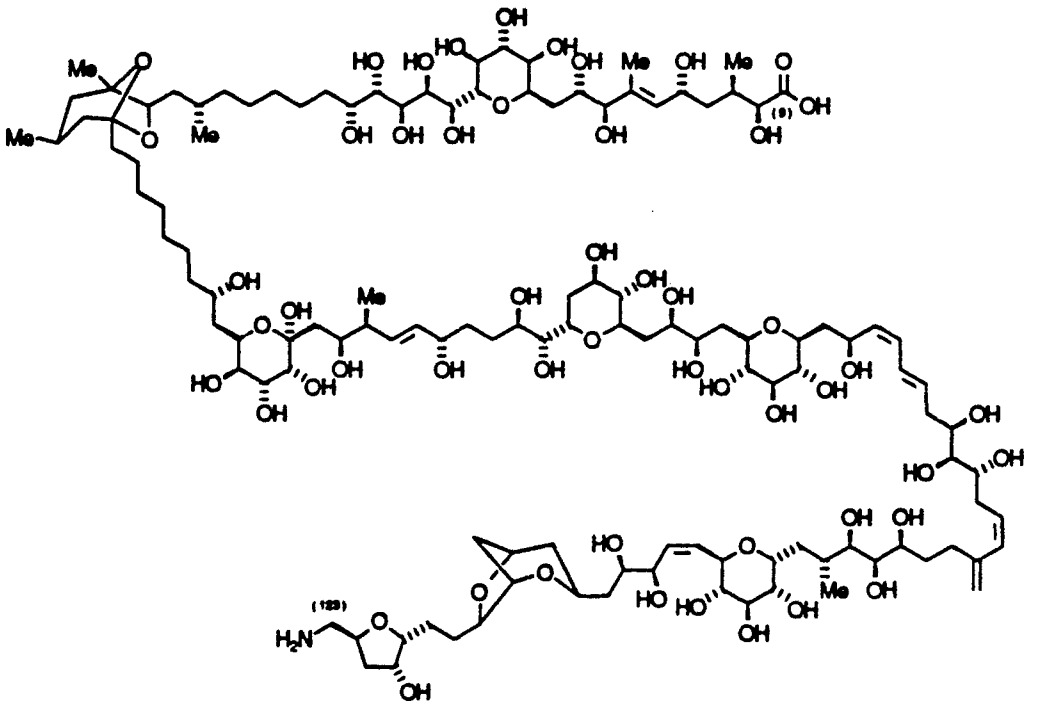


FIGURE 28. Sructure of Palytoxin Carboxylic Acid.

Upon our request, Dr. Uemura provided us with details of his isolation procedure for PTX-COOH and a 100 µg sample of the authentic material. As this quantity was too small to obtain an NMR, we were unable to independently verify the structure of this standard, however, it was fully cross-reactive in our PTX CIEIA and exhibited a different retention time than PTX in both TLC and HPLC. Uemura's PTX isolation procedure is almost identical to our own [7,18]. He claims that the PTX-COOH is present in his final purified PTX and that the two compounds can be separated by a simple HPLC procedure [53]. TLC analysis proved that the acid is not present in our purified PTX or an earlier-eluting fraction from the CM-Sephadex column which has a similar UV spectrum as PTX. It seemed unreasonable to us that the acid would be present in purified PTX as it would be expected to bind more tightly to the DEAE column than does PTX.

TLC of the crude extract from a fresh *P. toxica* sample (Isolation # 4.2 - see Table 3) confirmed the presence of a compound with the same R_f on both silica and amino HPTLC plates as the authentic PTX-COOH sample. The same compound was still detected following defatting and the XAD column. Since we believed that this acid was being lost on the DEAE anion exchange column, we split the sample into half and proceeded directly to the CM-Sephadex cation exchange step. This resulted in a clean

separation of PTX from the putative PTX-COOH. The other half of the material was processed in the same manner.

The fraction containing the putative PTX-COOH was still heavily contaminated with pigments and other compounds of higher R_f. There was far too much material to attempt purification by preparative TLC or HPLC. Further purification was attempted by organic phase gel filtration on Sephadex LH-20 (4:1 CH_3OH/H_2O). This resulted in a sample which appeared, by TLC, to contain mostly PTX-COOH. Analysis of the sample on a Showdex OHpak B804 HPLC column confirmed that it exhibited the same retention as Uemura's authentic PTX-COOH sample. The sample was further purified by preparative HPLC on the same column to yield ~6.4 mg of semi-purified PTX-COOH. HPLC analysis indicated that there were at least 3 other components in the mixture. Several attempts to obtain useful ^{1}H-NMR data on this mixture were not successful. The mixture was tested by CIEIA and found to contain less than 0.5 mg of antigenically reactive material. Based on this result and the encouraging results from an initial PTX hydrolysis experiment, we decided against further attempts to isolate PTX-COOH from *Palythoa* spp.

Because of our expectation that PTX-COOH should bind tightly to DEAE and therefore be easily removed from PTX, we became suspicious that the PTX-COOH in Uemura's purified PTX was due to hydrolysis during a later step of the isoltion. A 100 µg sample of PTX was treated with 10 equivalents of HCl in water at ambient temperature to determine if hydrolysis of PTX to PTX-COOH was feasible. The reaction was monitored by TLC after 1, 2, 6, 12, 24 and 48 hours. A spot matching the authentic PTX-COOH was detected after 6 hours, and conversion estimated to be ~50% after 24 hours. There was no apparent change in the ratio of PTX to PTX-COOH after another 24 hours but other decomposition products had begun to appear. UV indicated a significant reduction in the intensity of the absorption at 263 nm. This characteristic absorption of PTX is due to the unsaturated amide portion of the molecule.

Based on this encouraging result, we repeated the reaction with a sulfonic acid exchange resin as catalyst. Use of a resin should greatly simplify the workup. Although formation of PTX-COOH was observed by TLC early in the reaction, both PTX and the putative PTX-COOH disappeared shortly thereafter. No other degradation products were detectable. It is possible that both compounds bound irreversibly to this strong cation exchange resin.

Another HCl catalyzed hydrolysis reaction was performed on 200 µg of PTX and followed at 1 hour intervals by HPLC. No reaction was detected after 48 h with 10 equivalents of HCl. Additional 2-equivalent aliquots of HCl were added until hydrolysis was detected. A total of 14 additional equivalents were added. After incubating with the 24 equivalents of HCl for another 24 hours, only one peak, which co-elutes with Uemura's PTX-COOH, could be detected. This product also co-migrated with PTX-COOH on silica and amino HPTLC.

The PTX hydrolysis reaction was scaled up to 2 mg and followed hourly by HPLC. The chromatographic behavior of the

product, following workup on a C18 Bond elut column, was again identical to Uemura's authentic PTX-COOH on HPLC, silica TLC and amino TLC. A trace of PTX could be seen in the NMR spectrum, however, the interference due to large amounts of impurities made the NMR results inconclusive.

The reaction was repeated on 5 mg of PTX. Again, the chromatographic data looked promising, however, we could not obtain a good ^{1}H-NMR spectrum due to solubility problems. The material would initially dissolve in 85:15 CD_3OD/D_2O, but precipitate out again in ~15 min. This behavior did not make sense for PTX-COOH. Following recovery and dissolution in CH_3OH, TLC indicated the presence of several contaminants. An estimate of 5 mg of PTX-COOH in the sample was obtained by UV, assuming the same extinction coefficient as PTX at 233 nm. CIEIA, however, indicated that only 5% of this material is immunogenic with 73D3. Attempts to further characterize the putative PTX-COOH revealed that the bulk of the sample to be an unidentified plasticizer. The source of this plasticizer was traced to the inadvertent use of incompatible tubing to withdraw ethanol from a still. Attempts to recover the acid from the contaminating plasticizer failed.

After 12 hour exposure of another 5 mg of PTX to 25 equivalents of HCl, the reaction was carefully neutralized with the theoretical amount of NaOH and purified on a C18 Bond Elut SPE column. HPLC analysis of the reaction before neutralization, after neutralization and after the SPE column demonstrated that the workup did not alter the mixture. The chromatogram looked identical to that obtained in our earlier attempts to achieve this transformation. TLC analysis showed two major spots, PTX and another which exhibited similar R_f as the authentic PTX-COOH supplied by Uemura. These were separated by preparative HPTLC and the fractions quantitated by UV. CIEIA analysis of the second spot indicated < 10% immunogenicity in the putative acid sample. Reanalysis of the sample by TLC indicates that the R_f of the new spot was slightly lower then for authentic material. Based on this and the CIEIA data, we have concluded that the sample is not the desired acid.

At this point, we felt fairly confident that PTX-COOH was present, in low concentration, in both our crude *Palythoa* extracts and in the palytoxin hydrolysis reaction. We decided to investigate the use of immunoaffinity chromatography for isolating both PTX and PTX-COOH from these crude mixture. A 73D3 MAb-Sepharose immunoadsorbent (CNBr-activated) was prepared. One mg of PTX was applied to the immunoadsorbent column, of which 700 µg washed through. Treatment of the immunoadsorbent with 2M NaSCN to elute the bound PTX displaced only 29 µg. Immunoenzyme staining of Sepharose gel beads from the column clearly demonstrated the presence of a significant amount of bound PTX that had not been eluted by the 2M NaSCN. Additional agents for eluting PTX from the 73D3 MAb-Sepharose immunoaffinity column were investigated, but a suitable agent was not identified. This approach was therefore abandoned.

After reviewing the data from our unsuccessful attempts to prepare PTX-COOH from PTX or to isolate it from *Palythoa* spp., we

became more convinced that the acid was an artifact of Uemura's isolation and decided to make one last attempt to produce this compound by hydrolysis. A 1 g aliquot of CM-Sephadex (equilibrated with phosphate buffer, pH 4.5) was added to a 2 mg sample of PTX and incubated at ambient temperature. After 48 h, a trace amount of a new compound was detected which had the same R_f as authentic PTX-COOH on both amino and silica HPTLC. The amount of this new compound continued to slowly increase. After 1 week, there was no more apparent change in the ratio of PTX to the new material. The reaction mixture was applied to the top of the CM-Sephadex column and chromatographed by the usual PTX procedure. The new material eluted faster than PTX (as would be expected for the acid). One-hundred-seventy-eight µg of product, 368 µg of pure PTX and ~ 130 µg of a mixture of the two were recovered (quantities of the acid and mixed fractions based on UV absorbance at 233 nm using the reported extinction coefficient of PTX at this wavelength).

Careful HPTLC analysis demonstrated that the new product co-migrated with authentic PTX-COOH under both TLC systems. The material was ~ 50% as reactive in the CIEIA as authentic PTX-COOH. The cytotoxicity assay proved that the compound was ~ 50% as toxic as expected for pure PTX-COOH.

The evidence strongly suggested that the product was indeed the desired acid. The CM-Sephadex catalyzed hydrolysis was repeated with 15 mg of PTX. Following workup and purification, we obtained 3.3 mg of the the putative acid, 4.6 mg of recovered PTX and 1.5 mg of a mixed fraction. HPTLC cannot distinguish this product from the authentic PTX-COOH supplied by Uemura. The ^{1}H-NMR of the product proved that it was not PTX, as the C.7 proton signal at δ 7.8 was missing. The spectrum closely matches authentic spectra for PTX-COOH and palytoxin amide (PTX-C(O)NH_2) reported by Kishi, however, there were other olefin resonances not attributable to PTX-COOH. Careful inspection of NMR data revealed that these signals had been present as a trace impurity in the starting PTX. This spectrum alone was not conclusive evidence for the identity of the product. Several attempts were made to acquire FAB-MS data. The sensitivity of the University of Hawaii's mass specrometer was insufficient at this molecular weight range to acquire usable data.

Better ^{1}H-NMR data was obtained on the putative PTX-COOH sample using a special solvent supression technique. After careful examination of the data, it was decided that there was enough evidence that the product might be the acid to attempt conjugation to KLH.

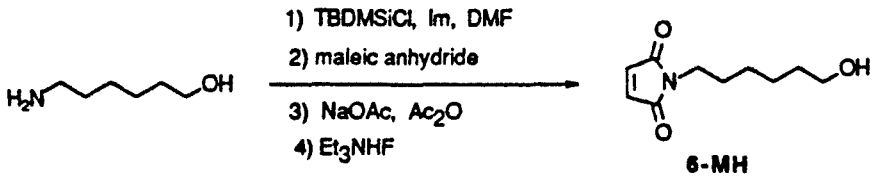
A portion of the putative PTX-COOH sample was subjected to conditions for conversion to the *N*-(2-nitrophenyl)sulfenamide (NPS-PTX-COOH). UV data for the purified product looked encouraging (no 263 nm absorption; 350 nm - NPS group). There was insufficient material to obtain an NMR. Conjugation of this material to KLH was attempted by EDC coupling. The product appeared to exhibit a slight increase in UV absorbance at 345 nm, as would be expected for the NPS group. Confirmation of this is difficult because KLH has an absorbance at this wavelength. The

NPS protecting group was removed from the conjugate via treatment with mercaptoethanol. Mice were immunized with this putative KLH-PTX-COOH conjugate.

Preparation of BSA-PTX-COOH was attempted via the same procedure used for KLH-PTX-COOH. Differential UV indicated a very low (<1 PTX/BSA) conjugation ratio. There was insufficient PTX-COOH to attempt a reprep of this conjugate.

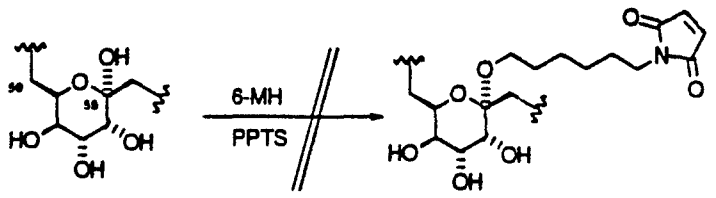
C.55 Ketal Strategy: Following a discussion with Professor Bob Armstrong (UCLA), a former Kishi student who worked on the PTX-COOH synthesis, we decided to pursue derivitization of PTX at the C.55 hemiketal position [54]. This work was originally undertaken in regard to the tritiation study and more explanation of the chemistry is presented in that section of this report.

A new linker, 6-maleimidohexanol (6-MH), was prepared as shown in Scheme 9. We attempted to form the mixed ketal with this alcohol at C.55 of PTX (Scheme 10).



SCHEME 9. Synthesis of 6-maleimidohexanol

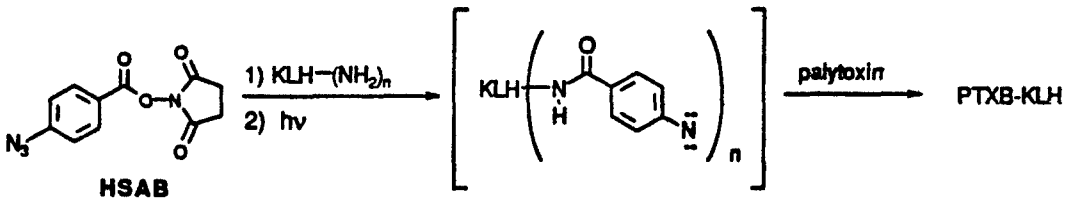
Although TLC and HPLC showed consumption of PTX when it was treated with 6-MH and pyridinium p-toluenesulfonate (PPTS) in DMF and in DMSO, no PTX-derived material bearing an intact maleimide ring could be detected in the NMR spectra.



SCHEME 10. Attempted ketalization of PTX with 6-maleimidohexanol.

Non-selective Conjugation Strategy: While the PTX-COOH and ketal approaches to a non-amino derivatized PTX hapten were being studied, we decided to pursue a backup strategy; non-regioselective conjugation utilizing hydroxysuccinimidyl 4-azidobenzoate (HSAB). The lysine residues of a protein react readily with HSAB to generate azidobenzamide residues. Exposure of the azido-derivatized protein to light generates reactive nitrene residues. If irradiation is done in the presence of a hapten, conjugation occurs via nitrene insertion into chemical bonds of the hapten. This strategy was successfully applied to synthesis of a metoclopramide immunogen [55]. While the site of coupling to PTX cannot be predicted, we thought it was possible

that the diene moieties of PTX may preferentially undergo cycloaddition to the nitrene. Thus, azidobenzoyl-KLH (AB-KLH) was prepared by treatment of KLH with HSAB. Photolysis of AB-KLH in the presence of NPS-PTX followed by removal of the NPS protecting group with mercaptoethanol gave an uncharacterized conjugate PTXB-KLH (Scheme 11). Mice were immunized with this material.



SCHEME 11. Non-selective Conjugation of Palytoxin and KLH via HSAB.

Palytoxin-73D3 Immune Complex: As an alternative hapten conjugate had not been synthesized by the start of Year 4, we developed another strategy for attempting to produce MAbs that would sandwich with MAb 73D3. One BALB/c and one Swiss-Webster mouse were immunized three times with an immune complex formed from 73D3 MAb and PTX mixed in a molar ratio of 100:1 (7 ng PTX per mouse) in Freunds Adjuvant. ELISAs on initial test bleeds from these mice, using microtiter plates wells coated with BSA-PTX-MCC, indicated a weak antibody response (1 in 80) in both animals. Hyperimmunization of these mice with [PTX-73D3 MAb] immune complexes was therefore continued. The Swiss-Webster mouse serum ELISA titer finally reached 1 in 640, while the BALB/c serum had a titer of only 1 in 100.

We had intended to give further injections to both of these mice after they had been rested for several weeks, when either a non-amino derivatised hapten was available or a suitable assay for detecting antibody to PTX epitopes other than the "73D3-like" MAb epitopes has been developed.

Immunoadsorbent Purification of antibodies from R7B7: As stated earlier, we unsuccessfully attempted to develop an immunoadsorbent column for the purification of PTX and PTX-COOH. Immunoenzyme staining of Sepharose gel beads from this column clearly demonstrated the presence of a significant amount of bound PTX that had not been eluted by the 2M NaSCN. The possibility of employing the PTX-73D3 MAb-Sepharose column for immunoaffinity purification, from R7B7 serum, of rabbit anti-PTX that might sandwich with 73D3 MAb immunoglobulin was investigated.

The R7B7 antibody that was immunoaffinity purified by this method exhibited minimal ELISA reactivity with BSA-PTX-MCC coated wells. In a direct CIEIA with AP-73D3 on BSA-PTX-MCC coated wells, however, considerable inhibition of reactivity was observed. These data strongly suggested that the immunoaffinity purified R7B7 antibody was closely associated with PTX that eluted with it from the 73D3-Sepharose (as an [R7B7-PTX] complex?), preventing its binding to BSA-PTX-MCC, and competing with BSA-PTX-MCC for binding to AP-73D3.

These observations indicated that the PTX-73D3 MAb-Sepharose column was not suitable for immunoaffinity purification of R7B7 antibody that will sandwich with 73D3 MAb. Because of this, the [PTX-73D3] immune complex immunized mice were not boosted, as the proposed MAb screening EIA could not be developed.

Screening of antisera from mice immunized with new conjugates: Bleeds from BALB/c and Swiss-Webster mice that have been primed and boosted with KLH-PTX-COOH and KLH-PTXB were tested by ELISA on microtiter wells coated with BSA-PTX-COOH. No reactivity was detected. No reactivity was obtained in an ELISA using AP-R7B7 on wells coated with BSA-PTX-COOH, suggesting that this coating antigen was not antigenic. When these bleeds were tested on wells coated with BSA-PTX-MCC, however, significant titers of anti-PTX reactivity were detected in all 3 Swiss-Webster mice. In an attempt to ascertain whether this response was against PTX-epitopes distant from the epitopes against which 73D3 MAb is directed, immunoglobulins, purified from these bleeds using Protein A-Sepharose, were coated onto wells and tested for their ability to sandwich PTX with AP-73D3. Unfortunately, sandwiching of PTX was not observed, suggesting that these new Swiss-Webster mice were responding to epitopes similar to, or close by, the epitopes against which 73D3 MAb is directed. At this point in the project, there was inadequate time remaining to further explore the synthesis of non-amino linked conjugates and the development of new MAbs was discontinued.

IMPROVEMENT OF ENZYME IMMUNOASSAYS FOR DETECTION OF PALYTOXIN IN BIOLOGICAL SAMPLES:

During Year 4 of this project, we investigated certain modifications to the direct sandwich ELISA and direct CIEIA to improve their performance, particularly when they were used for detecting palytoxin in biological samples.

Direct Sandwich ELISA:

The effect of substituting Protein A-Sepharose purified 73D3 MAb for saturated ammonium sulfate precipitated material as solid phase 'capture' antibody in the direct sandwich ELISA was investigated. Protein A-Sepharose purified MAb could be used at 25% the concentration of saturated ammonium sulfate precipitated MAb (2.5 µg/ml rather than 10 µg/ml), with no reduction in the system's sensitivity.

The possibility of adding sample and AP-73D3 conjugate to wells simultaneously rather than sequentially in the direct sandwich ELISA system was also investigated. This modification would reduce the total assay time by at least 60 minutes. Simultaneous addition of these reagents to wells caused no significant change in assay sensitivity. When samples contained palytoxin concentrations above 10-30 ng/ml however, significant inhibition of assay reactivity was observed. This may have been due to higher affinity AP-R7B7 antibodies in solution successfully competing with solid-phase 73D3 MAb for the same epitopes on PTX molecules, resulting in no free epitopes being available for 'capture' by 73D3. Further development of the simultaneous

sandwich ELISA system was therefore not pursued with the polyclonal and monoclonal antibodies that are currently available.

The use of the direct sandwich ELISA for detection of PTX in spiked human serum and plasma samples was investigated. Serum and plasma samples containing no PTX gave unacceptably high background absorbance values. These high background reactions were thought to be due to the presence of antibodies, in the human samples, that reacted with mouse and rabbit immunoglobulins. Indirect ELISAs on microtiter plate wells coated with mouse and rabbit immunoglobulins confirmed this possibility. Addition of normal species serum to diluent buffer did not absorb out this reactivity. It therefore appears that the sandwich ELISA, using the reagents available, is not suitable for use with human serum or plasma samples.

Direct CIEIA:

In view of the problems encountered with the direct sandwich ELISA, the suitability of the direct CIEIA for use with pooled human plasma was evaluated. This system detected PTX in spiked samples at concentrations down to 1.8 ng/ml (compared to 1.4 ng/ml in borate buffer), with no non-specific background or inhibition problems (Figure 29).

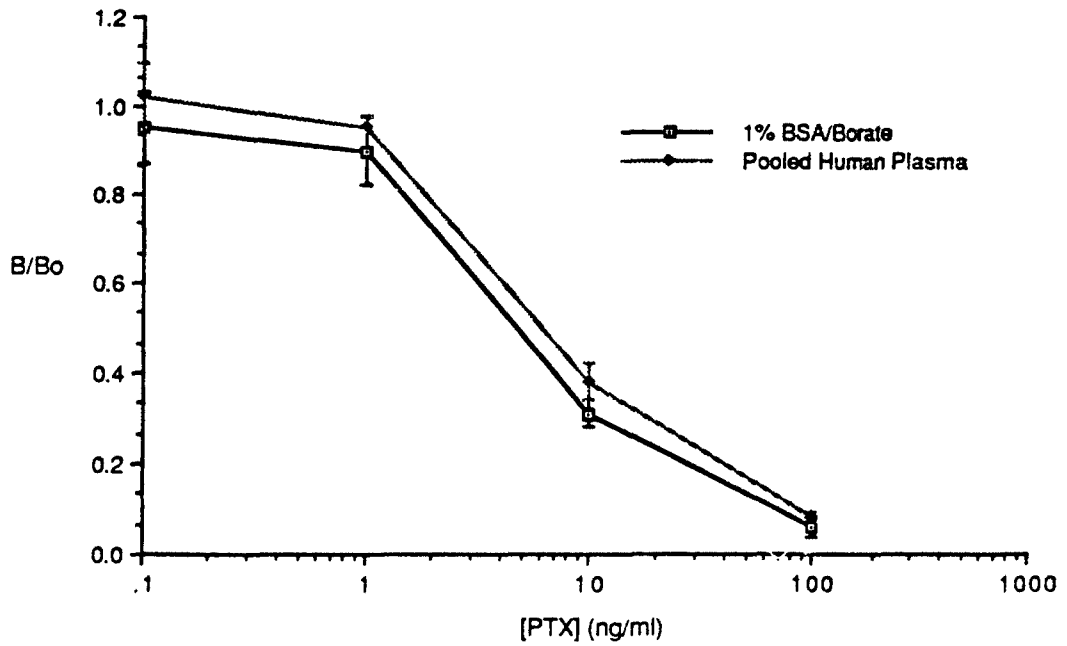


FIGURE 29. Comparison between the Performance of the Direct CIEIA with Palytoxin diluted in Pooled Human Plasma and 1% BSA-Borate Buffer.

DEVELOPMENT OF RAPID FIELD TEST FOR PTX DETECTION

Development of a rapid "field test" format for detection of palytoxin was discussed with Dr. John Hewetson during a visit by

James Raybould to Fort Detrick in March 1990. Dr. Hewetson indicated that this "field test" could be up to 10-fold less sensitive than the microtiter plate CIEIA system provided that it had a total test time of 4-6 minutes. We had originally intended to develop this system with a membrane device, previously used at HBG for a rapid detection system for tetrodotoxin. However, due to a considerable delay in the delivery of the membrane devices that we had hoped to use, we considered alternative formats and devices. The simplicity and low cost of latex agglutination systems prompted us to investigate the use of various sizes of latex particles coated with rabbit anti-PTX immunoglobulins, for rapid detection of PTX.

Initial development of this system utilized latex particles with diameters of 0.2, 0.5 and 0.8 µm (Bangs Laboratories, lot numbers L890524 [K020/868], L900907B [PS118C], and L900516C [K080/909] respectively) coated with Protein A-Sepharose purified R7B7 immunoglobulins at a optimized concentration of 216 µg immunoglobulin per mg latex This system detected 100-1000 ng/ml of PTX in a total assay time of 3-4 minutes. As our stock of R7B7 antibody became depleted, we substituted pooled Protein A-Sepharose purified immunoglobulins from rabbits 1,2 and 3 into the system. Further refinement of the system resulted in a direct latex agglutination test using latex particles coated with pooled immunoglobulin from rabbits 1,2 and 3, again at a concentration of about 216 µg per mg latex. Using either 0.2 or 0.8 µm latex particles, this system had a detection limit for palytoxin of 100 ng/ml with a total assay time of 1-2 minutes.

STABILITY OF PALYTOXIN ACTIVITY IN CRUDE *PALYTHOA* EXTRACTS

As a result of discussions at Fort Detrick on March 29, 1990, the stability of a crude 70% ethanol extract of *Palythoa tuberculosa* to elevated temperature and sunlight was investigated by two methods:

i) aliquots of undiluted extract were kept at 4 °C, room temperature (22-30 °C) and 37 °C for 18 hours, then tested, with controls, for antigenicity by direct CIEIA and for biologically active palytoxin in the <u>in vitro</u> cytotoxicity assay with EL-4 cells. There was no reduction in the concentration of antigenic or biologically active palytoxin in any test sample.

ii) a 1 in 100 dilution of extract (lower concentrations of palytoxin may be less stable) in a quartz cuvette was exposed to direct Hawaiian sunshine (air temperature on this day in April was 85 °F) for 2 hours, then tested, with controls, in the above <u>in vitro</u> cytotoxicity assay. This treatment caused no reduction in the level of toxicity of the diluted crude *Palythoa* extract.

These observations were communicated to USAMIID in a letter to Lt. Col. David Franz.

ADD-ON SUBPROJECTS

Lyngbya Majuscula Collection

Lyngbyatoxin A was isolated from the abundant, blue-green alga, *Lyngbya majuscula*. A total of six trips were made to Kahala Beach, O'ahu to collect *L. majuscula*. These collections are summarized in Table 15.

TABLE 15. Summary of *Lyngbya majuscula* collections at Kahala Beach, O'ahu

Date	Wet weight (kg)	Dry weight (g)
2/12/88	0.168	82.1
3/16/88	1.836	586.7
7/07/88	3.026	847.2
7/15/88	2.632	837.4
9/23/88	1.703	624.1
7/05/89	3.995	1588.1

Lyngbyatoxin A Isolation

Lyngbyatoxin A was isolated and purified via a procedure based on that published by Cardellina and Moore [32,33]. A flow diagram of the procedure followed is presented in Figure 30.

Six experiments were required to obtain a pure sample of lyngbyatoxin A and prove its identity by NMR. In the process, several minor changes were made to the reported chromatographic procedures. A major improvement in the process was use of centrifugal thin layer chromatography (Chromatotron™) for the final purification in experiments 7–10. Use of this technique instead of HPLC permitted a faster throughput of more material with no decrease in final purity of the toxin. The yields of lyngbyatoxin A from ten isolations are summarized in Table 16.

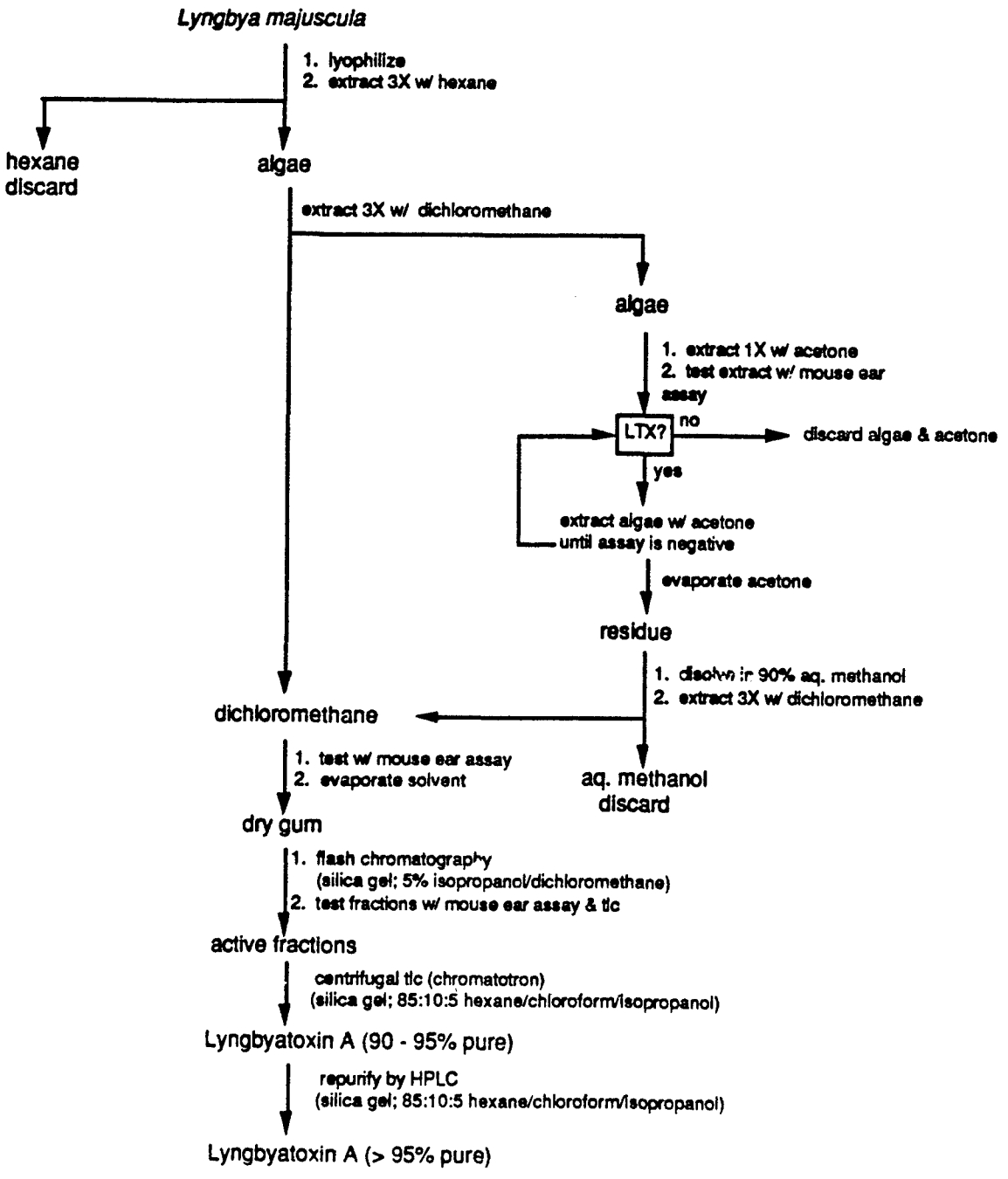


FIGURE 30. Isolation of Lyngbyatoxin A

TABLE 16. Summary of Lyngbyatoxin A Isolation.

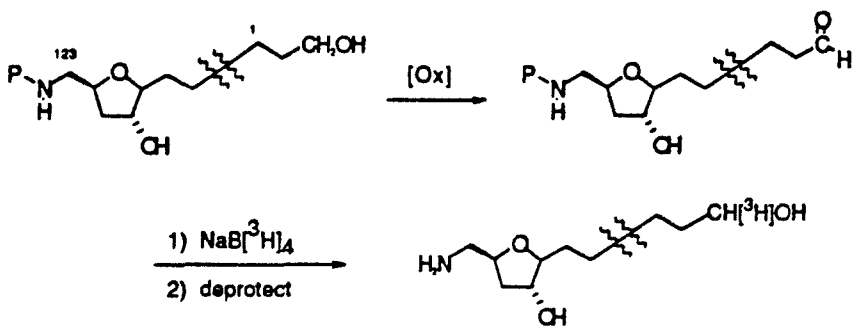
Experiment	Date	Dry Weight of Alga (g)	Crude Product (mg)	Final Product (mg)	Comments
1	2/12/88	82.1	177	------	Only a trace of LTX by NMR; delayed further work until freeze-dryer arrived
2	5/04/88	381.3	44	------	NMR ~30% LTX; all was lost on HPLC; possibly decomposed
3	5/20/88	205.4	1800	------	Technician left HBG during isolation; crude fractions were kept in freezer and combined on 9/13/88 for further work-up
4	7/11/88	847.2	44	------	~30% LTX by NMR
5	8/29/88	837.42	467		~50 % LTX by NMR
6	9/13/88	------	577	------	All LTX-containing fractions from exps.1-5 were combined and purified to ~60%
7	11/04/88	-----	577	53.3	The crude product from exp. 6 was repurified by HPLC to >95%.
8	1/26/89	624.1	249.1	100.6	Combined this batch and several impure fractions from earlier isolations; repurified on chromatotron to >95%.
9		785.3		35	20 mg of semi-purified fractions were added to the crude toxin of exp. 10 for purification. 90-95% pure.
10		802.79 g		55	90-95% pure.

Tritiation of Palytoxin:

During year 3 of this contract, we began studying methods for the tritium labeling of palytoxin. We anticipated that this would be an extremely challenging problem due to the complexity and chemically labile nature of the molecule. After consultation with researchers at Chemsyn Science Laboratories (CSL, Lenexa, Kansas), a company which offers custom tritiation services, we proposed a strategy for the synthesis of [^{3}H]PTX which was to involve selective oxidation of the C.1 hydroxyl group to produce palytoxin carboxaldehyde (PTX-CHO) followed by immediate reduction back to the alcohol with NaB[^{3}H]$_4$ (Scheme 12). CSL recommended against

trying catalytic hydrogen-tritium exchange because of the extreme reaction conditions of this method. They felt that the oxidation/reduction approach was the most reasonable way to proceed but only if dueterium incorporation could be accomplished with this sequence.

The proposed selective oxidation of the C.1 alcohol was not a trivial transformation. As stated in the earlier discussion of non-amino derivatised haptens, PTX presents a number of unique and difficult problems which severely restrict the types of reactions which can be employed. Most of the commonly used inorganic oxidants will cleave vicinal diols, indeed oxidative cleavage was one of the most important degradative reactions used to elucidate the structure of palytoxin [47]. Most of the commonly used organic oxidants have been developed for use in non-polar solvents and do not offer high selectivity for primary vs. secondary alcohols. We hypothesized that a modified version of the Swern oxidation [56] could be developed which would give the desired product. However, if oxidation of any of the secondary alcohols cold not be prevented, subsequent reduction would produce an epimeric mixture of palytoxins. Amino group protection was also required due the the facile reaction of amines with aldehydes to form Shiff's bases. A final consideration was that aldehydes are often not stable, therefore the workup had to be quick and produce a crude aldehyde preparation suitable for immediate reduction.



SCHEME 12. Proposed Oxidation/Reduction Strategy for Tritium Labeling of Palytoxin.

Initial attempts to oxidize *N*-AcPTX to the aldehyde looked encouraging. Two variations of the Swern conditions were attempted on ~ 1 mg of material. Treatment of *N*-AcPTX with trifluroacetic anhydride/DMSO/TEA & oxalyl chloride/DMSO/TEA at -60 °C in a mixture of CH_2Cl_2 and DMSO gave carbonyl containing products as evidenced by a positive 2,4-dinitophenylhydrazine test on TLC. The oxalyl chloride procedure was then attempted on 2 mg of N-AcPTX in CD_2Cl_2/d_6-DMSO so that we could analyze the mixture for formation of the desired aldehyde by NMR. No evidence of an aldehyde signal could be detected in the NMR spectrum. TLC indicated the formation of carbonyl containing products as evidenced by a positive 2,4-dinitophenylhydrazine test. This

2,4-DNP positive spot has the same R_f as the starting material in every solvent system tried. There are three possible explanations for this result:

a) the aldehyde is present as a hydrate or as a hemiacetal formed with one of the PTX hydroxyl groups.

b) the aldehyde was not formed and the 2,4-DNP positive spots are due to oxidation of one or more of the secondary hydroxyls to a ketone.

c) The orange spots are due to carbonyl containing decomposition products.

It soon became obvious that the major practical concern for this approach was detection of a successful oxidation of the molecule. Resolution of the product from PTX by chromatography was doubtful due to the minute difference in overall polarity. The desired aldehyde would almost certainly exist as an internal hemiacetal, thus the expected aldehyde proton signal at δ9-10 would be shifted into a complex region of the NMR spectrum and probably could not be assigned. Carbohydrate chemists have long used high voltage paper electrophoresis in sodium bisulfite buffer as an analytical tool for carbohydrate derived aldehydes [57]. The negatively charged bisulfite addition product is formed in situ and migrates toward the anode. We arranged access to an old high voltage paper electrophoresis apparatus at the University of Hawaii with the intention of using this instrument to monitor the oxidation reactions. Unfortunately, this instrument could not be made operable and no other instrument was available.

We had anticipated the use of fast-atom-bombardment mass spectroscopy (FAB-MS) as a tool for determining deuterium incorporation. Several attempts to obtain FAB-MS spectra of PTX at the University of Hawaii were unsuccessful. Their instrument proved to have insufficient sensitivity and resoultion at this molecular weight range. Since Kishi reported FAB-MS data for his synthetic PTX-COOH [8], we planned to look for available time on MS instruments at mainland institutions, however, because of the probable high cost, we first wanted to verify that this method had the potential to solve our problem. Therefore, mass spectral simulations were run for PTX, d_1-PTX and a 50:50 mixture of the two on the system at UH. This data is presented in Figure 31. Inspection of these predicted fragmentation patterns reveals that the difference between the FAB-MS for the pure compounds and a 50:50 mixture of the two is slight. They become indistinguishable when one considers that these simulations assume no noise in the data and that the signal to noise ratio reported by Kishi for PTX-COOH was ≈ 2. Simulation results for the incorporation of two deuteriums are even messier. Thus, we were forced to conclude that FAB-MS was not a viable analytical method for our needs.

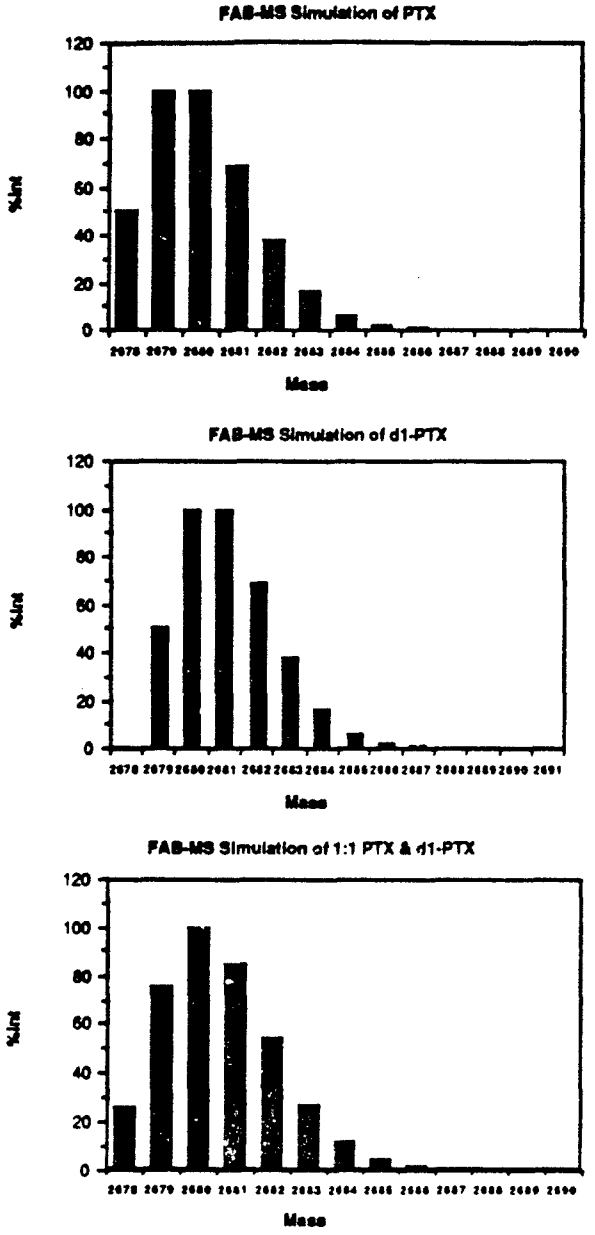
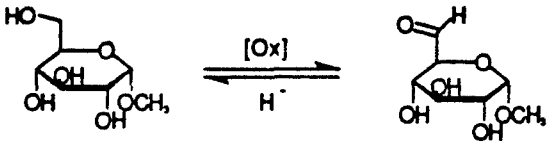


FIGURE 31. Mass Spectral Simulations of PTX and d_1-PTX

Dr. Walter Niemczura, the director of the UH NMR/MS facility, recommended that we look for ^{13}C-^{2}H coupling in the ^{13}C-NMR spectra. Substitution of a single deuterium causes the observed signal (in the fully proton-decoupled spectra) to change from a singlet to a triplet whose lines are of equal intensity. Additionally, because of the loss of the Nuclear Overhauser Effect, the total signal intensity is much lower. The obvious drawback to this approach was the amount of compound and time needed to acquire usable spectra. Several attempts to acquire

^{13}C-NMR data with a 600 MHz instrument on 5-10 mg of PTX failed to give a spectrum of adequate quality for this approach to succeed.

At this point, the only viable analytical method which we had was to visualize TLC plates with carbonyl-specific reagents such as 2,4-DNP and purpald (Aldrich). Therefore we decided to optimize oxidation/reduction reaction conditions on a simplier model system, methyl-α-D-glucopyranoside (**3**), with the intention of subjecting a small amount of PTX to the optimized sequence with tritiated borohydride (Scheme 13). The reaction would than have been analyzed by HPLC with both radiochemical and UV detection to see if any of the label was incorporated.



3 **6**

SCHEME 13. Oxidation/Reduction Model Study

Swern oxidation of **3** produced the aldehyde (**6**) as evidenced by subsequent reduction with $NaBH_4$ in EtOH to regenerate **3** as the major product. However, this material was contaminated with several diastereomeric alcohols, resulting from oxidation of one or more of the secondary alcohols followed by reduction of the keto group. In addition, there are minor amounts of several regio- and diastereomeric methylthiomethylethers, a known by-product of the Swern oxidation. The overall recovery of **3** is only ~ 50%, too low for successful tritiation of PTX. Several variations of reaction conditions and workup procedures failed to improve this result.

We also investigated catalytic oxidation (O_2, Pt/C) as a possible solution to this problem. Extensive use of catalytic oxidation in carbohydrate chemistry has demonstrated a very strong preference for primary versus secondary hydroxyl, the usual solvent is water and workup simply involves filtration of the catalyst [58,59]. Subsequent reduction with $NaBH_4$ could be performed in the resulting aqueous solution. Drawbacks of the procedure are the slow rate (typical reaction half-life is ~ 12 h), deactivation of the catalyst by amino groups, and highly variable yields. Some examples of direct oxidation of amino-sugars are known, however, protection of amino groups is usually required.

Conversion of **3** to **6** occurred in variable yields ranging from 30-90% after 3 days in the presence of platinum on carbon (PT/C) under an oxygen atmosphere. Attempts to standardize this reaction were unsuccessful. Attempted oxidation of NPS-PTX under these conditions gave no detectable reaction, possibly due to poisoning of the catalyst by the sulfur atom of the NPS group. However, the same result was obtained *N*-acetylpalytoxin. The same disappointing result was observed when *N*-Ac-PTX was treated with O_2 in the presence of elemental platinum (produced by hydrogenation

of PtO_2). When elemental ruthenium was used as the catalyst, TLC indicated that there was slight formation of an overlapping spot which stained with 2,4-dinitrophenylhydrazine. *N*-Ac-PTX does not stain with this reagent, which is known to react specifically with carbonyl groups. After 48 hours, the catalyst was removed by filtration and $NaB[^2H]_4$ was added. The new spot was no longer visible in the TLC. We had hoped to determine if deuterium incorporation had occurred by FAB-MS, however, the spectral simulations, which were proceeding at this time, demonstrated that FAB-MS would not be capable of distinquishing PTX fron d_1-PTX.. Several repetitions of the ruthenium catalyzed oxidation of *N*-AcPTX failed to produce the 2,4-DNP reactive product detected in the earlier run.

We then revised our strategy and attempted to effect oxidation of one or more of the allylic hydroxyl groups of PTX. We expected that detection of the enone product(s) would be possible by ^1H-NMR and UV. Reduction with $NaB[^3H]_4$ would give an epimeric mixture of palytoxins Oxidation of allylic alcohols with $NiSO_4$-$K_2S_2O_8$ is reported to work well in a biphasic H_2O-CH_2Cl_2 mixture [60]. The oxidant is believed to be nickel peroxide and the oxidation occurs in the aqueous phase. This seemed to be a good candidate for the oxidation of PTX.

Initial results of a model study with D-glucal (**7**) looked encouraging (Scheme 14). A UV-active spot with slightly higher R_f on silica gel was observed. An attempt was made to convert the product to the diacetate so as to simplify purification and characterization, however, the compound decomposed under the acetylation conditions. A French group has reported that this conversion has been effected with Fetizon's reagent (Ag_2CO_3-celite) [61]. Their procedure was repeated to provide an authentic sample of the enone. This material proved to not be identical to the product of the Nickel peroxide reaction.

7

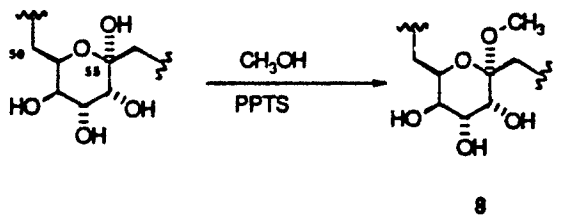
SCHEME 14. Model system for allylic oxidation.

One attempt was made to oxidize N-AcPTX under the nickel peroxide conditions. This resulted in complete degradation of the molecule within several minutes. At this point, we concluded that selective oxidation of PTX was an unachievable goal with the resources at hand and decided to investigate our backup strategy, the partial reduction of olefin bonds with $[^3H]_2$.

There are no reports of the relative reactivity of the 8 alkene bonds in PTX. Ideally, we would have prefered to reduce only one of these bonds. Complete hydrogenation to hexadecahydro-PTX has been reported [47]. Since one cannot accurately measure the consumption of a gas on a μmol scale, we attempted to optimize

the reaction by varying the time. Because of the lack of an adequate chromatographic or mass spectral analytical technique, we were forced to do the reaction on a sufficient scale for NMR analysis. The reaction was performed on 15 mg of *N-Ac*PTX in 95% ethanol. At 5, 15, 30, 60, and 240 minutes, the catalyst was allowed to settle and 1/5 of the original solvent volume (~3 mg of the *N-Ac*PTX) was removed and evaporated to dryness. NMR revealed that reduction of the double bonds was facile (no detectable olefin signals after 30 minues) and there was no apparent selectivity. TLC indicated the presence of many compounds with overlapping R_f values. The total number of possible diastereomeric regioisomers would be (# regioisomers) X (# combinations of reduction from top and bottom of each double bond) = 8! X 8! = 1,625,702,400. Given this preliminary result, we concluded that this approach was not feasible.

Our last strategy was based on a discussion with Dr. Bob Armstrong (UCLA), one of Dr. Kishi's former students who participated in the PTX-COOH synthesis [54]. This involved conversion of the C.55 hemi-ketal to a ketal (**8**) by treatment of PTX with a weak acid and methanol (Scheme 15). If this product retained biological activity and was chemically stable towards hydrolysis, we planned to repeat the preparation with tritiated methanol.



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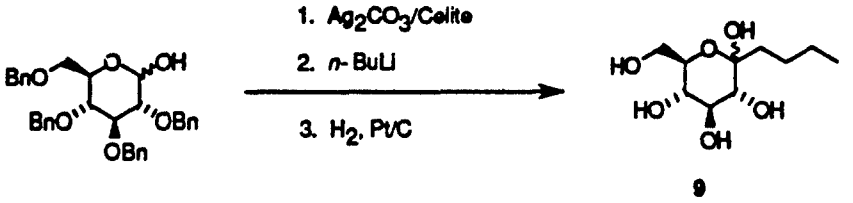
SCHEME 15. Proposed ketaliztion of PTX.

A 2 mg sample of PTX was treated with 0.1 equivalents of pyridinium *p*-toluenesulfonate in anhydrous methanol. TLC indicated formation of a new compound with an R_f slightly higher than PTX. Three possible $-OCH_3$ singlets were observed in the NMR spectrum, however, due to the complexity of the spectrum, we could not ascertain if the reaction had worked. HPLC analysis of the reaction product gave a very broad peak with the same retention time as PTX. This would indicate that there are several components in the sample. Thus we have decided to optimize the reaction with a model system. Synthesis of the model is shown below.

Hemiketal **9** was chosen as a reasonable model system. Unfortunately, we were unable to accomplish the final debenzylation to prepare **9** from 2,3,4,6-tetra-*O*-benzyl-D-glucopyranose (obtained from Pfansteihl) as shown in Scheme 16.



9

SCHEME 16. Attempted synthesis of a ketal model compound.

Since at this time, we had identified the ketalization of PTX with 6-maleimidohexanol (6-MH) as a possible approach to a non-amino derivatized conjugate (see above), we decided not to pursue preparation of **9**. A successful ketalization with 6-MH would have been easily detected by NMR due to the lack of interfering peaks in the maleimide region of the spectrum. Thus, this hapten synthesis would serve as a model for the methanol ketalization. The contract ended without success in this endeavor.

One additional strategy for the production of a tritium labeled palytoxin derivative would be the acylation of a radiolabled alcohol or amine with palytoxin carboxylic acid. Our contacts at CSL told us that treatment of a carboxylic acid with diazomethane in $[^3H]_2O$ produces the corresponding methyl esters with high specific activity. Unfortunately, we were not able to obtain sufficiently well characterized palytoxin carboxylic acid to pursue this approach.

DELIVERABLES

The deliverables due for this project and the status of each at the end of the contract are presented in Table 17

TABLE 17. Project Deliverables

Item	Description	Status
0001AA	200 mg purified palytoxin	54 mg delivered 1/18/88 54 mg delivered 3/21/89 100 mg delivered 3/14/89 Handling Instructions and Data Sheets included as Appendices W, X, and Y
0001AB	Immunogens 5 mg - if requested	COR telecon with D. Vann 6/20/89; not needed since useful antibodies have been produced.
0001AC	Procedural reports for coupling item 0001AB	Included in Annual and Final Reports
0001AD	Antisera (5 ml) derived from testing of item 0001AB	5 ml antiserum R7B7 sent 2/22/89 10 ml antiserum R7B7 + 10 ml antiserum fronm rabbits 1, 2, & 3 sent 11/13/90
0001AE	Hybridoma cell line with affinity to palytoxin (up to 5)	4 vials of 73D3.2.1 cell line ~ 10^7 cells sent 9/7/88 3 vials of 73D3.2.1 cell line ~ 10^7 cells sent 3/28/89 Monoclonal hybridoma lines from cell lines 88G9 and 89F9 can be provided upon request, though these MAbs exhibit properties similar to 73D3
0001AF	Reagent for palytoxin determination (enough for 10,000 samples)	35 ml mouse ascites fluid sent 9/7/88 10.5 ml mouse ascites fluid sent 2/22/89 10 ml mouse ascites fluid sent 10/22/90 55 ml total of 73D3 has been supplied. This is sufficient for many more than 10,000 sample determinations. Coating antigen will be supplied if requested.
0001AG	Immunoassay procedural for palytoxin developed using ELISA formats	Included in Annual and Final reports.

TABLE 17. Continued

Modification No. P70001		
0001AH	Lyngbyatoxin A (1-10 lots of 50 mg each)	50 mg delivered 11/21/88 100 mg delivered 3/21/89 Data Sheet included as Appendix Z (~90 mg semipurified LTX A available awaiting disposition decision)
0001AJ	Report on the method of collection, isolation, purification, yields , and efficiency of item 0001AH	Included in Final Report
Modification P90004		
0001AK	100 mg highly purified palytoxin	70 mg delivered 1/10/90 30 mg delivered 2/26/91
0001AL	Supporting analytical data for item 0001AK	Data sheets included as Appendix AA
0001AM	Report of chemical methods for tritium labeling of palytoxin	Experiments were not successful. Full discussion in Final Report
0001AN	High specific activity tritiated palytoxin (10-15 mg)	Synthesis was unsuccessful.
0001AP	Excess palytoxin from tritiation of item 0001AP	Over 200 mg of palytoxin have been used in failed palytoxin tritiation attempts.

DISCUSSION

The overall goals of this BAA project, as stated in the original proposal, were:

(i) to collect *Palythoa* soft coral, and isolate and purify a total of 200 mg of palytoxin for delivery to USAMRIID,

(ii) to produce high affinity polyclonal and monoclonal antibody preparations against palytoxin hapten-protein conjugates, and

(iii) to develop sensitive immunoassays for detecting and quantitating palytoxin in biological samples.

Change orders were added to the original proposal, which involved isolation and purification of 50-500 mg of lyngbyatoxin A from *Lyngbya majuscula*, delivery of an additional 100 mg of

that our recovery of palytoxin from the crude coral extracts is ~30%, an excellent recovery for such an involved isolation procedure. A total of 208 mg of palytoxin was delivered to USAMRIID over the first two years of this contract. Palytoxin haptens were synthesized via the amino terminus of the molecule using 2 different linker molecules, and conjugated to proteins, for use as immunogens for antibody production, and as solid phase-coating antigens for immunoassay development. High titer rabbit polyclonal antisera and high affinity mouse monoclonal antibodies against palytoxin were produced; certain monoclonal antibodies proved to be capable of neutralizing the toxicity of palytoxin both in vivo in a mouse model system and in vitro in a cell culture cytotoxicity system. Three indirect enzyme immunoassay systems were developed during Year 1 of the project, and preparation of alkaline phosphatase conjugates of polyclonal antibodies, monoclonal antibodies and palytoxin itself during Year 3, enabled development of three further direct enzyme immunoassay systems. Extensive characterization and validation studies on these systems proved that the direct CIEIA system using alkaline phosphatase labelled 73D3 MAb, which had a detection limit for palytoxin of about 1 ng/ml, was the method of choice for determination of palytoxin levels in biological matrices. As a result of discussions between USAMRIID and the project P.I. during 1990, an additional, extremely rapid and simple latex agglutination test was developed, with a total assay time of 1-2 minutes and a detection limit for palytoxin of 100 ng/ml. Two of the three objectives in the change orders were also achieved. A total of 150 mg of lyngbyatoxin A (3X the minimum required) and an additional 100 mg of palytoxin were delivered. Unfortunately, the third objective, tritiation of palytoxin, was unsuccessful due to the high level of difficulty associated with synthetic organic chemistry involving the palytoxin molecule.

The rabbit antiserum indirect CIEIA developed during Year 1 was clearly not the immunoassay method of choice. The slope of the dose response curves for palytoxin detection were very shallow and were variable in terms of degree of inhibition at a moderately high concentrations of palytoxin. Evidence was obtained that the BSA-PTX-PDP coating antigen was unstable, though the reason for this instability was not determined.

All the MAbs that were produced against palytoxin appeared to be directed against the same epitope, or against epitopes that are close together on the PTX molecule. As all these MAbs exhibited similar properties, 73D3 was selected for EIA development as it has been most extensively characterized.

Anti-palytoxin monoclonal antibody 73D3 proved to be suitable for use in immunoassays. The MAb performed well as indicator antibody in an indirect CIEIA system, as AP-labelled conjugate in a direct CIEIA system, and as solid phase capture antibody in both indirect and direct sandwich ELISAs and a direct CIEIA with AP-labelled palytoxin. Monoclonal antibody 73D3 was found to be effective at neutralizing the in vitro cytotoxic activity of palytoxin. The success of the preliminary collaborative study of in vivo therapeutic usefulness of this antibody conducted with Dr. John Hewetson points to a potentially exciting and useful

application of this agent. The hybridoma clone that produces MAb 73D3, was delivered as frozen cells to USAMRIID, where they were successfully thawed and grown in culture. The required quantities of this MAb were delivered to USAMRIID as ascites fluid and as purified immunoglobulins.

The 73D3 MAb indirect CIEIA has excellent characteristics in terms of detection limit for palytoxin, the slope of the titration curve, and between-assay reproducibility. Studies on the specificity of the system indicated that active and inactive forms of palytoxin were the only agents showing significant reactivity. None of the other marine toxins or other compounds tested were significantly reactive. It is striking that palytoxin could be treated by procedures that reduced its biological activity by 100,000-fold or more (as determined by the in vitro cytotoxicity system), while its serological activity was only reduced 10-fold,at most. Levine, et al., [38] reported a similar, though less dramatic, observation.

During the latter part of Year 1, an indirect sandwich ELISA was also developed. The principle underlying a sandwich ELISA requires that antibodies detect two distinct epitopes on the analyte molecule. MAb 73D3 was partially purified to increase its specific activity, and used as solid phase (microtiter plate well) 'capture' antibody. R7B7 rabbit polyclonal antibody was used as the second antibody. Detection of the antibody-antigen-antibody sandwich was achieved with commercially available alkaline-phosphatase labelled goat anti-rabbit immunoglobulin conjugate. Sandwich immunoassays are considered to be highly specific for an intact antigen since a positive signal can only result when two specific binding events have taken place.

Since the same palytoxin immunogen, prepared via the palytoxin amino group, had been used to elicit both the antibody preparations available at that time, we knew that at least two separate epitopes existed distal to the amino terminus. At that time, it appeared possible that through hapten formation at alternative conjugation sites, more epitopes could be defined. This possibility offered a rationale for our subsequent search for haptens linked at primary or secondary hydroxyl groups on palytoxin.

Preliminary spike and recovery experiments, performed with the indirect CIEIA using crude extracts, during Year 1, yielded palytoxin concentrations that were not significantly different from expected values. In the example presented in Figure 13, matrix interference was not detected when different dilutions of a crude extract were spiked with known amounts of purified palytoxin. The slopes of the lines were consistent with the expected value of 1.0 and the increments between extracts of different dilutions conformed to predicted behavior. However, as shown in Figure 14, non-ideal behavior was observed with other extracts.

During Year 2, a major effort was made to characterize and validate both the indirect CIEIA and the indirect sandwich ELISA. In particular, extensive experiments were designed and performed

to determine why measurements of calculated palytoxin concentration exhibited the phenomenon of 'non-parallelism'. The summary of results presented in the relevant section of this report was distilled from one of three similar sets of experiments.

The data generated from the initial spike and recovery experiments with the indirect CIEIA, during Year 1, indicated that the variation in the palytoxin concentrations recovered from crude extract dilutions spiked with known amounts of palytoxin were similar in magnitude (about 50%) to that of the IC_{50} values of indirect CIEIA standard curves derived with purified palytoxin.

The experiments performed during Year 2 were of a different type, in which unspiked crude extracts were serially diluted, assayed by the indirect CIEIA and indirect sandwich ELISA systems, and estimates of the palytoxin content of the undiluted sample calculated. In the results from these experiments, striking and consistent non-parallelism was seen, where the estimate of palytoxin concentration in a particular crude extract increased with sample dilution. When crude extract samples were diluted to the point where their B/B_0 values fell on the linear portion of the standard curve (normally a B/B_0 value between 0.15 and 0.85), several dilutions of the same crude extract gave different values for palytoxin concentration, with no way of ascertaining which value was the correct one (Figure 16 and Table 10). A similar "non-parallelism" phenomenon was observed when the same extract dilutions were tested by the indirect sandwich ELISA (Figure 17 and Table 11)

In an attempt to discover ways to understand this phenomenon, several hypotheses were generated and examined. One hypothesis is that the matrix interferes with antigen-antibody reactions, either decreasing or increasing reactivity nonspecifically. A nonspecific decrease in antigen-antibody reactions would show up in the CIEIA format as results which give higher calculated values at low dilutions--this is the opposite of the observed results. As shown in Figure 17, the indirect sandwich ELISA format exhibits a phenomenon of non-parallelism similar to that seen with the Indirect CIEIA. This is inconsistent with the hypothesis that the matrix is capable of nonspecifically augmenting antigen-antibody reactions. Thus, simple matrix interference mechanisms appear inadequate to explain the results.

Another hypothesis is that palytoxin forms stable aggregates at a critical concentration and upon dilution the aggregates fall apart. Thus a 1/100 dilution of an extract could contain 10 units in the aggregate form. When diluted to 1/1000, the aggregates could fall apart giving rise to 5 monomers from each aggregate. Either an aggregate or a monomer would be scored as a "unit" in the immunoassay. The following table illustrates this hypothetical situation.

A selection of disruptive agents, such as detergents and surfactants were added to the assay system. They had no effect and therefore there was no support for this hypothesis.

Dilution	Actual Conc.	Number of Units	Calculated conc (= Units X Dil.)
1/100	500	10	1000
1/1000	50	5	5000

If aggregation were occurring, it is possible that the aggregates could be constructed in such a way that a particular palytoxin epitope (e.g. the epitope against which MAb 73D3 was reactive) would be present several times in the aggregate unit. The biotinylated antibody indirect sandwich ELISA was developed to test this hypothesis. The control experiments indicated that this experiment should have detected any aggregated forms of palytoxin that had multiple epitopes reactive with MAb 73D3 (if they were present). Sandwiching of palytoxin between two 73D3 MAb molecules was not observed in this system, there is no support for the "palytoxin aggregate" hypothesis.

As neither matrix interference nor the aggregate hypothesis were supported, an explanation for the non-parallel behavior of the indirect CIEIA and indirect sandwich ELISA systems observed at that time with crude extracts, could not be explained.

As noted in the "Results" section of this Final Report, indirect enzyme immunoassay systems are less cost-effective than direct enzyme immunoassay systems as they require an additional reagent, an additional incubation, and an additional washing step. The use of analyte-specific monoclonal or polyclonal antibodies directly conjugated to enzyme overcomes these disadvantages. By avoiding the need for a species-specific enzyme labelled antibody conjugate, the assay cost is reduced in terms of both reagents and time, and in addition, any risk of non-specific reactivity between naturally occuring antibodies in the anti-species conjugate and other components of the system is avoided. At the beginning of Year 3 of this project, a decision was therefore made to prepare purified antibody immunoglobulin preparations, produce conjugates directly labelled with alkaline phosphatase (AP), and develop direct enzyme immunoassay systems.

Affinity chromatography using discontinuous pH gradient elution from Protein A-Sepharose has been extensively used for commercial purification of MAbs from ascitic fluid and spent hybridoma culture medium. The method was therefore evaluated for purification of 73D3 MAb. Data obtained indicated that this method produced a greater yield of MAb with a higher specific activity than purification using Protein G. The method has therefore been adopted for routine purification of 73D3 MAb.

After investigating several conjugation procedures, directly labelled AP-MAb conjugates have been prepared from Protein A purified 73D3 MAb, using the heterobifunctional coupling reagent Sulfo-SMCC. This method has previously been used for successful preparation of commercial AP-MAb conjugates. As noted in the "Results" section, considerable time was spent during the final two years of this project attempting to optimize procedures for conjugating AP to both 73D3 and R7B7 immunoglobulins.

A direct CIEIA using AP-73D3 on microtiter plate wells coated with BSA-PTX-MCC was successfully developed, which required one step less than the indirect CIEIA, resulting in a more rapid assay and cost savings in terms of time and reagents. This direct CIEIA had a total test time of 2 hours and a sensitivity level equal to or greater than the indirect CIEIA (Table 13).

A conjugate of PTX directly labelled with AP was also prepared. The AP-PTX conjugate retained its antigenicity with respect to binding by 73D3 MAb, and exhibited high AP enzyme activity. A direct CIEIA using this conjugate on microtiter plate wells coated with 73D3 MAb was also developed (Table 13). This system had a sensitivity range similar to the indirect CIEIA, but like the direct CIEIA with AP-73D3 MAb described above, involved one step less, resulting in cost savings in time and reagents.

Both the newly developed CIEIA systems suffer from the disadvantage that they require PTX hapten conjugate, synthesis of which requires extraction of *Palythoa* and purification of PTX, both of which are expensive and potentially hazardous. Development of a direct sandwich ELISA system (Table 13) would have many advantages. A direct sandwich ELISA would require suitable antibodies only, and avoid the need for PTX hapten conjugate. During Year 4, successful production of AP-R7B7 conjugates enabled development of a MAb-capture, polyclonal antibody-detector, direct sandwich ELISA. As shown in Table 13, this direct sandwich ELISA was as rapid as the direct CIEIAs, and exhibited a similar detection limit for palytoxin. We had hoped to produce further high affinity MAbs that would be suitable for development of a MAb-capture, MAb-detector direct sandwich ELISA, but the difficulty of the chemistry involved in synthesizing other palytoxin haptens prevented achievement of this goal.

When the five EIA systems were used to ascertain the PTX content of the Okala Island *Palythoa tuberculosa* extracts once again during Year 3, all five systems gave similar results (with the exception of certain extracts, for instance #3). Further, the concentrations determined in these experiments were (again with certain exceptions) similar to those obtained when the extracts were previously tested by the indirect sandwich ELISA and indirect CIEIA (See Tables 10 and 11 in this Final Report).

It is interesting that in the EIA data on the Okala *Palythoa* extracts obtained during Year 3 of the project, the "non-parallelism" effect reported during Years 1 and 2 was seen only with certain extracts in the indirect sandwich ELISA, and not at all in the indirect CIEIA or either of the direct CIEIAs. One possible reason is that these extracts contained a wide range of PTX concentrations. In Year 3, in order to ensure that the B/B_0 or OD values for two or three dilutions of each extract fell within the middle region of the linear portion of the standard curve for that particular assay (between 0.3 and 0.7), each extract was titrated through a broad dilution series (1 in 6.25, 1 in 25, 1 in 100, 1 in 400, 1 in 1600, 1 in 6400, and 1 in 25,600 - see Figure 27). In the indirect assays performed during Year 2 on these same extracts, only four standard dilutions of most extracts (100, 400, 1600, 6400) were tested (see Figures 16 and 17),

resulting in B/B_0 values between 0.15 and 0.85 having to be used to calculate PTX concentrations. Comparison of Figure 27 with Figures 16 and 17 suggests that the PTX values determined for dilutions of certain extracts that were reported to be exhibiting "non-parallelism" in the Year 2 assays, were actually PTX concentrations calculated from the non-linear portions of standard curve for that particular assay. However, this observation cannot be used to explain the extracts that exhibited the "non-parallelism" phenomenon in the experiments performed during Year 3 (e.g. extracts 2 and 3 in the indirect sandwich ELISA). In conclusion, the cause of the "non-parallelism" phenomenon is not clear; the phenomenon did appear to occur less frequently, however, with the direct enzyme immunoassay systems developed during Years 3 and 4 of the project, than with the indirect systems developed during Years 1 and 2.

During the final year of this project, we made a decision to concentrate on the direct sandwich ELISA and direct CIEIA systems as the systems of choice for palytoxin detection. We therefore investigated certain modifications to each system with the intention of improving their performance, particularly when they were used for detecting palytoxin in biological samples. These experiments showed us that even though we felt the direct sandwich ELISA was the more suitable system for use by USAMRIID because it did not require palytoxin hapten-conjugate, this system suffered from considerable matrix interference when used with human plasma samples. The direct CIEIA was not subject to this matrix interference, and detected PTX in spiked pooled human plasma at concentrations down to 1.8 ng/ml (compared to 1.4 ng/ml in borate buffer). We therefore recommend the direct CIEIA system for use by USAMRIID.

The stability studies performed on the AP-labelled R7B7 and 73D3 conjugates provided interesting data. AP-R7B7 conjugate in 50% glycerol exhibited no reduction in reactivity after storage for 39 weeks at 0 °C. Storage of AP-MAb conjugate in 50% glycerol did not significantly increase its stability. The longest stability trial that was possible during this project, once AP-MAb conjugates had successfully been prepared, indicated that AP-73D3 conjugate (stored in 0.01 M Tris-HCl buffer, pH 6.8 containing 0.1 M NaCl, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 0.5 g/l NaN_3 and 1% BSA) was stable to storage at 4 °C for at least 49 weeks (355 days). Accelerated stability studies on lyophilized AP-73D3 conjugate indicated that this reagent was stable to storage at 37 °C for at least 3 months. Using the conversion commonly used in the industry that one month stability of a reagent at 37 °C suggests approximately one year stability at 4 °C, this accelerated stability data suggests that lyophilized AP-73D3 conjugate might be stable for up to 3 years when stored at 4 °C. These data on the stability of liquid AP-conjugates stored at 4 °C and lyophilized AP-conjugates stored at 37 °C demonstrate our ability to produce stable enzyme-antibody conjugates.

Development of the latex test for detection of palytoxin deserves comment. The requirement for a simple, rapid immunoassay with this level of sensitivity, was discussed with Dr. John

Hewetson, by James Raybould, the PI for this project during its final two years, during a visit to Fort Detrick in March 1990. As the latex agglutination system was developed during the final months of this project, validation with biological samples of the type that might be found in the field would be required to prove its utility. However, the latex agglutination system clearly demonstrates our capability for developing rapid immunoassays of this type that might be amenable to field use.

During the same visit referred to in the previous paragraph, the stability of palytoxin to light and heat (and therefore its potential threat as a biological weapon) was also discussed. The experiments performed as a result of these discussions clearly demonstrate the excellent stability of a crude 70% ethanol extract of *Palythoa tuberculosa* to elevated temperature and sunlight.

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PERSONNEL

The following individuals, all of whom are present or former members of HBG's Immunology and Chemistry groups, contributed to the success of this project.

Immunology

T.J.G. Raybould, Ph.D., Principal Investigator (12/89 - 5/91) - directed enzyme-antibody conjugations and stability studies; development and validation of all direct immunoassay systems using enzyme labelled polyclonal antibody, monoclonal antibody and palytoxin conjugates; development of rapid latex-agglutination assay.

Douglas C. Vann, Ph.D., Principal Investigator (3/87 - 7/89) - directed initial palytoxin hapten conjugations; immunizations and antiserum screening; development and validation of all indirect enzyme immunoassay systems; discovery and investigation of "non-parallelism" phenomenom.

Gary S. Bignami, M.S. - responsible for in vitro cytotoxicity assays, hybridoma fusions, and MAb production.

John K. Uno, M.S. - assisted in immunoassay development; hapten-protein conjugation, Years 1-2.

Ramona M. Visnak, B.S. - assisted in immunoassay development, Year 2.

Samantha Simpson, B.S. - assisted in immunoassay development, Years 3-4.

Jilanne B. Byrnes, B.S. - assisted in immunoassay development, Year 4.

Kent E. Harada, B.S. - assisted in latex agglutination assay development.

Chemistry

Paul G. Grothaus, Ph.D., Chemistry Group Leader (1/88 - 5/91), Interim Principal Investigator (7/89 - 12/89) - primary responsibility for palytoxin and lyngbyatoxin A isolation; linker, hapten, and hapten-protein conjugate synthesis, purification and characterization; studies for tritiation of palytoxin.

Navzer D. Sachinvala, Ph.D., Original Chemistry Group Leader (3/87 - 12/87) - primary responsibility for palytoxin isolation; linker and hapten, synthesis, purification and characterization.

ToBun Cheung, B.S. - responsible for palytoxin isolation, purification and characterization, Year 1.

Carolyn B. Lazo, B.S. - responsible for palytoxin and lyngbyatoxin A isolation, purification and characterization; linker and hapten synthesis, purification and characterization, Years 2-4.

Jeanette Hermosa, B.S. - responsible for palytoxin and lyngbyatoxin A isolation, purification and characterization, Year 3.

Melissa Churley, M.S. - responsible for linker and hapten synthesis, purification, and characterization, Year 4.

Special acknowledgement to Professor Richard E.Moore (University of Hawaii) for consultation regarding palytoxin isolation and characterization, Professor Marcus A. Tius (University of Hawaii) for consultation regarding hapten synthesis and design of tritiation chemistry. and Dr. Walter Niemczura (University of Hawaii) for consultation regarding NMR and MS experiments and data.

APPENDIX A

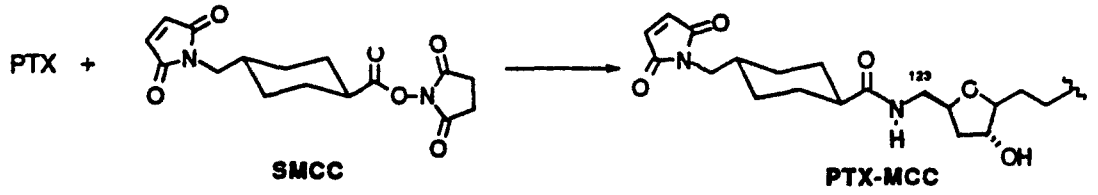
Synthesis of *N*-(2-pyridyldithio)propionylpalytoxin (PTX-PDP)

A solution of *N*-(2-pyridyldithio)proprionate (SPDP) (4.7 mg, 15 µmol) in DMSO (100 µl) was added to a solution of palytoxin (8.1 mg, 3.0 µmol) in 0.1 M phosphate buffer, pH 7.5 (1.9 ml). After being stirred for 3.5 hours at room temperature, the reaction mixture was extracted with CH_2Cl_2 (3 X 0.5 ml). The aqueous phase was applied to a 1 ml C-18 BondElute SPE column, which had been previously washed extensively with methanol and then with water. The effluent from the SPE column was collected and reapplied to the top of the column. The SPE column was wahed 3X with water to remove the buffer salts and then with 80% aqueous ethanol to elut the crude product. The ethanol was removed in vacuo to yield the crude hapten.

The hapten was purified by ion-exchange chromatography on CM Sephadex-C25 with 0.02M phosphate buffer, ph 4.6, UV detection 254 nm. The hapten fractions were combined and desalted on a C-18 SPE column as above. Following evaporation of the ethanol, the sample was dilutid in water (2-5 ml) and lyophilized to yield 4.9 g (60% yield) pure PTX-PDP as a fluffy white solid.

APPENDIX B

Synthesis of *N*-(4-(*N'*-maleimidomethyl)cyclohexane-1-carboxoylpalytoxin
(PTX-MCC)



A solution of succinimidyl 4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (sulfo-SMCC) (6.2 mg, 15 µmol) in DMF (100 µl) was added to a solution of palytoxin (8.1 mg, 3.0 µmol) in 0.1 M phosphate buffer, pH 7.5 (1.9 ml). After being stirred for 8 hours at room temperature, the reaction mixture was extracted with CH_2Cl_2 (3 X 0.5 ml). The aqueous phase was applied to a 1 ml C-18 BondElute SPE column, which had been previously washed extensively with methanol and then with water. The effluent from the SPE column was collected and reapplied to the top of the column. The SPE column was wahed 3X with water to remove the buffer salts and then with 80% aqueous ethanol to elut the crude product. The ethanol was removed in vacuo to yield the crude hapten.

The hapten was purified by ion-exchange chromatography on CM Sephadex-C25 with 0.02M phosphate buffer, ph 4.6, UV detection 254 nm. The hapten fractions were combined and desalted on a C-18 SPE column as above. Following evaporation of the ethanol, the sample was dilutid in water (2-5 ml) and lyophilized to yield 4.4 g (54% yield) pure PTX-MCC as a fluffy white solid.

APPENDIX C

KLH-PTX-PDP Conjugation
June, 1987

Materials:

1. 25 mM borate buffer, pH 9.0
2. 0.05 M sodium phosphate buffer, pH 6.6, 1 mM EDTA
3. KLH (dialyzed in borate buffer)
4. 2-iminothiolane
5. Sephadex G-25 column equilibrated with at least 2 bed volumes of phosphate buffer
6. dithiodipyridine (DTDP) and BCA assay reagents
7. palytoxin dithiopyridyl hapten (PTX-PDP)

Method:

1. Measure OD_{280} of diluted KLH aliquot to estimate KLH concentration. (Extinction coefficient = 2.02 $(mg/ml)^{-1}$)
2. Mix KLH with a 50-fold molar excess of 2-IMT in borate buffer. React for 1 h. at room temperature, with stirring.
3. Apply thiolated KLH (KLH-SH) to G-25 column. Elute with phosphate buffer, collecting 20 drop fractions. Read OD280 of fraction, pool desired fractions into tared tube.
4. Perform DTDP (Appendix I) and BCA (Appendix H) assays, calculate KLH concentration and mole -SH per mole KLH.
5. Mix KLH-SH with a 5-fold molar excess of PTX-PDP, relative to the net KLH thiolation. React for 1 h. at room temperature, with stirring.
6. Dialyze the KLH-PTX-PDP against four changes of 500 ml phosphate buffer.
7. Transfer KLH-PTX-PDP to tared tube. Perform DTDP and BCA assays. Calculate KLH concentration and moles -SH per mole KLH. The decrease in measurable sulfhydryls per KLH molecule is an indirect estimate of the degree of PTX conjugation to KLH.

APPENDIX D

BSA-PTX-PDP Conjugation
June, 1987

Materials:

1. 25 mM borate buffer, pH 9.0
2. 0.05 M sodium phosphate buffer, pH 6.6, 1 mM EDTA
3. BSA
4. 2-iminothiolane
5. Sephadex G-25 column equilibrated with at least 2 bed volumes of phosphate buffer
6. dithiodipyridine (DTDP) and BCA assay reagents
7. palytoxin dithiopyridyl hapten (PTX-PDP)

Method:

1. Mix BSA with a 50-fold molar excess of 2-IMT, in borate buffer. React for 1 h. at room temperature, with stirring.
2. Apply thiolated BSA (BSA-SH) to G-25 column. Elute with phosphate buffer collecting 16-20 drop fractions. Read OD_{280} of fractions, pool desired fractions into tared tube.
3. Perform DTDP (Appendix I) and BCA protein (Appendix H) assays, calculate BSA concentration and mole -SH.
4. Mix BSA-SH with a 5-fold molar excess of PTX-MCC, relative to the net BSA thiolation. React for 1 h. at room temperature, with stirring.
5. Dialyze the BSA-PTX-PDP against 500 ml phosphate buffer, changing buffer four times.
6. Transfer BSA-PTX-PDP to tared tube. Perform DTDP and BCA assays. Calculate BSA concentration and moles -SH per mole BSA. The decrease in measurable sulfhydryls per BSA molecule is an indirect estimate of palytoxin conjugation to BSA.

APPENDIX E

KLH-PTX-MCC Conjugation
March, 1990

Materials:

1. 25 mM borate buffer, pH 9.0
2. 0.1 M sodium phosphate buffer, pH 6.6, 1 mM EDTA
3. KLH (dialyzed in borate buffer)
4. 2-iminothiolane
5. Sephadex G-25 column equilibrated with at least 2 bed volumes of phosphate buffer
6. dithiodipyridine (DTDP) and BCA assay reagents
7. palytoxin maleimide hapten (PTX-MCC)

Method:

1. Measure OD_{280} of diluted KLH aliquot to estimate KLH concentration. (Extinction coefficient = 2.02 $(mg/ml)^{-1}$)
2. Mix KLH with a 50-fold molar excess of 2-IMT in borate buffer. React for 1 h. at room temperature, with stirring.
3. Apply thiolated KLH (KLH-SH) to G-25 column. Elute with phosphate buffer, collecting 20 drop fractions. Read OD280 of fraction, pool desired fractions into tared tube.
4. Perform DTDP (Appendix I) and BCA (Appendix H) assays, calculate KLH concentration and mole -SH per mole KLH.
5. Mix KLH-SH with a 5-fold molar excess of PTX-MCC, relative to the net KLH thiolation. React for 1 h. at room temperature, with stirring.
6. Dialyze the KLH-PTX-MCC against four changes of 500 ml phosphate buffer.
7. Transfer KLH-PTX-MCC to tared tube. Perform DTDP and BCA assays. Calculate KLH concentration and moles -SH per mole KLH. The decrease in measurable sulfhydryls per KLH molecule is an indirect estimate of the degree of PTX conjugation to KLH.

APPENDIX F

BSA-PTX-MCC Conjugation
March, 1990

Materials:

1. 25 mM borate buffer, pH 9.0
2. 0.1 sodium phosphate buffer, pH 6.6, 1 mM EDTA
3. BSA
4. 2-iminothiolane (2-IMT)
5. Sephadex G-25 column equilibrated with at least 2 bed volumes phosphate buffer
6. dithiodipyridine (DTDP) and BCA assay reagents
7. palytoxin-MCC hapten

Method:

1. Mix BSA with a 50-fold molar excess of 2-IMT, in borate buffer. React for 1 h. at room temperature, with stirring.
2. Apply thiolated BSA (BSA-SH) to G-25 column. Elute with phosphate buffer collecting 16-20 drop fractions. Read OD_{280} of fractions, pool desired fractions into tared tube.
3. Perform DTDP (Appendix I) and BCA protein (Appendix H) assays, calculate BSA concentration and mole -SH.
4. Mix BSA-SH with a 5-fold molar excess of PTX-MCC, relative to the net BSA thiolation. React for 1 h. at room temperature, with stirring.
5. Dialyze the BSA-PTX-MCC against 500 ml phosphate buffer, changing buffer four times.
6. Transfer BSA-PTX-MCC to tared tube. Perform DTDP and BCA assays. Calculate BSA concentration and moles -SH per mole BSA. The decrease in measurable sulfhydryls per BSA molecule is an indirect estimate of palytoxin conjugation to BSA.

APPENDIX G

BSA-PTX Conjugation
March, 1990

Materials:

1. 0.1 sodium phosphate buffer, pH 7.4
2. BSA
3. palytoxin
4. EDC

Method:

1. Prepare a solution of BSA (25 mg/ml) in phosphate buffer.
2. Add BSA to a 5-fold molar excess of palytoxin. Add 10-fold molar excess of EDC. React for 12 h. at 4 °C, with stirring.
3. Transfer reaction mixture to a Centricon™ 30. Spin at 4000 rpm at 4 °C until solution is concentrated to ~ 200 µl.
4. Add PBS to the concentrate and spin down to ~ 200 µl again. Repeat buffer exchange 3X. Adjust volume to 1 ml.
5. Transfer PTX-MCC-BSA to tared tube.
6. Measure OD_{280} of diluted BSA-PTX aliquot to estimate protein concentration.
7. Establish working dilution in appropriate EIA.

APPENDIX H

BCA Protein Assay
(Pierce Chemical Co. Protocol)

Materials:

1. BCA working reagents: 50 parts Reagent A + 1 part Reagent B.
2. Buffer for diluting standards and samples.
3. Protein standard: BSA at 1 mg/ml.

Method:

1. Make up assay samples in duplicate. To separate tubes containing 100, 80, 60, 40, 20, and 0 µl buffer to separate tubes at dilutions estimated to be in range of 0.2 to 0.8 mg/ml.
2. Add 2.0 ml BCA working reagent to each tube. Mix. Incubate 30 min. in 37° C water bath. Cool tubes to room temperature in tap water bath. Measure OD_{562}.

APPENDIX I

Determination of Sulfhydryl Groups (DTDP Assay)

(Grassetti, D.R.; Murray, J.F. *Arch. Biochem. Biophys.* **1967**, *119*, 41-49)

Materials:

1. 2 mM 2,2'-dithiopyridine (DTDP). Weigh out 10 mg of DTDP into an Eppendorf tube, add 100 µl DMF to dissolve. Add this to 22.6 ml phosphate buffer, pH 7.
2. Phosphate buffer, pH 7 (Can use PBS or 0.05 M phosphate, pH 6.6, 1 mM EDTA).

Methods:

1. Make up samples in duplicate, diluted in phosphate buffer. To separate tubes containing 0.5 ml buffer, native proteins, thiolated proteins or conjugated proteins, add 0.5 ml 2 mM DTDP, mix.
2. Incubate samples 15 min. at room temperature. Read OD_{343} using "buffer and DTDP only" as blank.
3. Calculate [-SH] in samples using the following formula:
   Molar extinction coefficient of 2-thiopyridine at 343 nm = 7060
   Equation 1. Calculation of free sulfyhryl groups
   $[-SH] = 2 \times (OD_{343})/7060\ M^{-1}$

Note:

This assay will not give reliable results with peroxidase or KLH, both of which have significant optical absorbance in the region of 343 nm. A method involving separation of protein from the low m.w. 2-thiopyridine by Centricon (Amicon) ultrafiltration can be used. Using alkaline phosphatase or BSA, this assay gives reasonably reliable results for protein concentrations of 0.2 to 0.8 mg/ml.

APPENDIX J

Primary Indirect ELISA Screen for Antibodies to Palytoxin
March, 1990

Materials:

1. Immulon 2 microtiter plates (Dynatech)
2. BSA-PTX-PDP, BSA-PTX-MCC or other BSA-PTX coating antigen
3. 50 mM Sodium Phosphate, pH 7.0, 0.15 M NaCl (PBS)
4. PBS containing 0.05% (v/v) Tween-20 (PBS-T)
5. 50 mM Tris HCl, pH 7.0, 0.15 M NaCl, 0.05% (v/v) Tween 20 (TBS-T)
6. Bovine Serum Albumin (BSA)
7. Alkaline Phosphatase goat anti-rabbit Ig conjugate(or alkaline phosphatase goat anti-mouse Ig conjugate for monoclonal antibody screen)
8. *p*-nitrophenylphosphate (pNPP) (Sigma 104, 5 mg tablets)
9. Alkaline Phosphatase substrate buffer:
   25 mM Trizma base, pH 9.5
   0.15 M NaCl
   5 mM $MgCl_2$
   0.02% (w/v) NaN_3

Method:

1. Coat Immulon 2 microtiter plates with BSA-PTX-PDP, BSA-PTX-MCC, or other BSA-PTX coating antigen, 100 µl/well, at ** µg/ml in PBS for 1 h. at room temperature.
   ** coating antigen concentration must be determined in advance for each batch. Normally, twice the minimum saturating concentration is used.
2. Wash three times with PBS-T.
3. Block wells of microtiter plate with 200 µl/well of 1% BSA in PBS for 1 h. at room temperature.
4. Wash three times with PBS-T.
5. Add 100 µl/well of the preparation being tested for anti-PTX antibody, titrated in 1% BSA in PBS. Incubate for 1 h. at room temperature.
6. Wash three times with PBS-T.
7. Add 100 µl/well goat alkaline phosphatase goat anti-rabbit Ig conjugate(or alkaline phosphatase goat anti-mouse Ig conjugate for monoclonal antibody screen), diluted to appropriate concentration with 1% BSA in PBS. Incubate 1 h. at room temperature.
8. Wash four times with TBS-T.
9. Add 200 µl/well 1 mg/ml pNPP diluted in alkaline phosphatase substrate buffer, pH 9.5.
10. Read on dual wavelength absorbance mode (reference wavelength = 690 nm = filter 8 on Titertek; sample wavelength = 414 nm = filter 3 on Titertek).

APPENDIX K

Affinity Purification of Murine Monoclonal Antibody from Ascitic Fluid by Discontinuous pH Gradient Elution from Protein A-Sepharose
March, 1990

Materials:

1. Staphylococcal Protein A-Sepharose-CL-4B (Pharmacia)
2. Econocolumn (Biorad)
3. Buffer #1: 1.45 M glycine-NaOH, 3 M NaCl, pH 8.9
4. Buffers #2-5: 0.04 M sodium citrate, 0.02 M NaCl, pH 6.0, 5.0, 4.0, and 3.2, respectively.
5. Neutralizing buffer: 0.5 M sodium phosphate, pH 7.7
6. UV monitor
7. Fraction collector
8. Ultrafiltration cells and filters
9. Ascitic fluid containing monoclonal antibody of interest.

Method:

1. Fully hydrate and equilibrate Protein A-Sepharose with Buffer 1 in a glass Econocolumn at 4 °C.
2. Dilute ascites fluid in 3 parts Buffer 1 and apply to column at a flow rate of 1-5 ml/min..
3. Wash column with Buffer 1 until entire unbound peak is eluted.
4. Collect run-through and successive protein peaks by fraction collector or by pooling an entire buffer elution.
5. Elute bound material successively with Buffers 3, 4, and 5, into 1/4 volume neutralizing buffer.
6. Alternatively bound material may be eluted in one step using buffer 4 only.
   [Collecting individual buffer fractions as in step 5 provides a better purification of monoclonal antibody from non-specific antibody.]
7. Eluted fractions or pools may be assayed for antigen-specific monoclonal antibody by ELISA.
8. Concentrate appropriate eluates using ultrafiltration to 1-5 mg/ml. Store at -20°C.
9. Antibody concentration may be estimated by measuring OD_{280}. A mouse IgG solution at 1 mg/ml = 1.44 Absorbance units (OD_{280}).

APPENDIX L

Alkaline Phosphatase-73D3 Conjugation
(based on methods of Ishikawa et al.[43])
March, 1990

Materials:

1. Alkaline Phosphatase (AP)(Sigma)
2. Sulfo-SMCC: Sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (Pierce)
3. 50 mM sodium borate buffer, pH 7.6, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$
4. 1.0 ml Sephadex G-50 (fine) spin column
5. Dialysis tubing
6. 0.1 M Tris-HCl, pH 7.0, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$ (Buffer T)
7. 73D3.2.1 anti-palytoxin monoclonal antibody (protein A purified)
8. 0.1 M sodium phosphate buffer, pH 6.5
9. *S*-acetylmercapto-succinic anhydride (SAMSA)
10. Dimethylformamide (DMF)
11. 0.1 M EDTA
12. 0.1 M Tris-HCl, pH 7.0
13. 1 M hydroxylamine-HCl, pH 6.5
14. 0.1 M sodium phosphate buffer, pH 6.0
15. Centricon 30 microconcentrators (Amicon)
16. DTDP assay reagents
17. 10 x 1 cm Sephadex G-200 column
18. 10 mM Tris-HCl buffer, pH 6.8 containing 0.1 M NaCl, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$ and 0.5 g/l NaN_3 (Eluting buffer)
19. IEC benchtop centrifuge

Method:

1. Dialyze AP against borate buffer at room temperature with two buffer changes.
2. React the AP with a 25 fold molar excess of sulfo-SMCC in borate buffer for 30 min. at 30°C.
3. Apply 100 µl aliquots of the AP-MCC to 1 ml Sephadex G-50 (fine) spin columns equilibrated with Buffer T. Centrifuge 2 min.
4. Collect AP-MCC filtrates and concentrate on a Centricon 30 equilibrated with buffer T.
5. Concentrate the protein A purified 73D3 and exchange the buffer with 0.1 M sodium phosphate, pH 6.5 using a Centricon 30 equilibrated with the same buffer. Centrifuge 20 min. at 3000 x g and collect retentate. Estimate protein concentration by measuring OD_{280}.
6. React the 73D3 with a 100 fold molar excess of SAMSA to thiolate the antibody. Incubate 30 min. at room temperature with stirring. Add 0.02 ml 0.1 M EDTA, 0.1 ml 0.1 M Tris-HCl, and 0.1 ml hydroxylamine-HCl. Incubate 4 min at 30°C.
7. Transfer thiolated 73D3 to a Centricon 30 equilibrated with pH 6 phosphate buffer, fill to 2 ml with pH 6 phosphate buffer, and centrifuge 20 min. at 3000 x g to remove unreacted SAMSA. Collect retentate in tared tube.

8. Perform the DTDP assay (Appendix I) on the thiolated 73D3 to determine the mole -SH to mole antibody ratio.
9. React thiolated 73D3 with a 5 fold molar excess of AP-MCC. Incubate overnight at 4°C with stirring.
10. Transfer the 73D3-AP reaction mix to an Eppendorf tube and microcentrifuge for 5 min. at top speed.
11. Apply the supernatant to a Sephadex G-200 column equilibrated with Eluting Buffer and elute 60 drop fractions, monitoring the absorbance at 280 nm.
12. Test selected fractions by ELISA and pool positive fractions.

APPENDIX M

Alkaline Phosphatase-Palytoxin Conjugation
March, 1990

Materials:

1. Palytoxin-MCC hapten (PTX-MCC) resuspended in water.
2. SPDP: *N*-succinimidyl 3-(2-pyridyldithio)propionate (in EtOH) (Pierce)
3. Alkaline Phosphatase (Sigma)
4. Centricon 30 microconcentrator units (Amicon)
5. 0.1 M sodium acetate buffer, pH 4.5
6. IEC benchtop centrifuge
7. Dithiothreitol (DTT) in sodium acetate buffer
8. 0.1 M sodium phosphate buffer, pH 6.5
9. DTDP assay reagents
10. 1.0 ml Sephadex G-25 spin column equilibrated with 0.1 M sodium phosphate buffer, pH 6.5
11. Beckman DU-7 spectrophotometer

Method:

1. React AP with a 25 fold molar excess of SPDP. Bring reaction volume up to 1.0 ml with sodium phosphate buffer, pH 6.5 and do not allow the final EtOH concentration to exceed 1%. Incubate 30 min. at room temperature with stirring.
2. Transfer reaction mixture to a Centricon 30 equilibrated with acetate buffer, fill to 2 ml with acetate buffer, and centrifuge at 3000 x g for 20 min. Repeat centrifugation 3 times with excess buffer and collect retentate in a tared Eppendorf tube.
3. Add DTT solution in sodium acetate buffer to a final concentration of 25 mM and incubate 30 min. at room temperature with stirring.
4. Transfer reaction mixture to a Centricon 30 equilibrated with sodium phosphate buffer, fill to 2 ml with pH 6.5 phosphate buffer, and centrifuge 20 min. at 3000 x g. Repeat 3 times to exchange the buffer completely.
5. Apply the reaction mix to a Sephadex G-25 spin column (100 µl/1ml column) and centrifuge 5 min at 700 x g. Pool the filtrates in a tared tube.
6. Determine the number of -SH groups on the AP by using the Sulfhydryl Group Determination Assay (see Appendix I).
7. React the thiolated AP with a 5:1 molar ratio of PTX-MCC to thiolated AP. Incubate overnight at 4°C.
8. Transfer the conjugate mixture to a Centricon 30 equilibrated with sodium phosphate buffer, fill to two ml with sodium phosphate buffer, and centrifuge 3000 x g for 20 min. Repeat 6 times to remove unreacted PTX-MCC and collect retentate in tared tube.
9. Test the conjugate by ELISA.

APPENDIX N

Indirect CIEIA for Palytoxin
March, 1990

Materials:

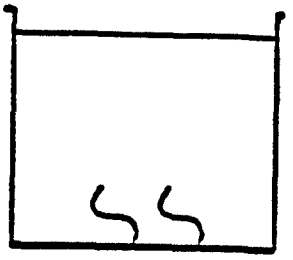
1. Immulon 2 microtiter plates (Dynatech)
2. BSA-PTX-MCC coating antigen
3. 50 mM Sodium Phosphate, pH 7.0, 0.15 M NaCl (PBS)
4. PBS containing 0.05% (v/v) Tween-20 (PBS-T)
5. 50 mM Sodium Phosphate, pH 7.0, 0.15 M NaCl, 0.5 mM $Na_2B_4O_7$ (PBS-B)
6. 50 mM Tris HCl, pH 7.0, 0.15 M NaCl, 0.05% (v/v) Tween 20 (TBS-T)
7. Bovine Serum Albumin (BSA)
8. Alkaline Phosphatase goat anti-mouse Ig conjugate (or alkaline phosphatase goat anti-rabbit IgG if anti-palytoxin rabbit antibody preparation is used)
9. *p*-nitrophenylphosphate (pNPP) (Sigma 104, 5 mg tablets)
10. Alkaline Phosphatase substrate buffer:
    25 mM Trizma base, pH 9.5
    0.15 M NaCl
    5 mM $MgCl_2$
    0.02% (w/v) NaN_3
11. Protein A-Sepharose purified 73D3 anti-palytoxin monoclonal antibody or 73D3 containing ascites fluid (or anti-palytoxin rabbit antibody preparation)
12. Palytoxin (stored at -20°C in 50% EtOH)

Method:

1. Coat Immulon 2 microtiter plates with PTX-MCC-BSA, 100 µl/well, at ** µg/ml in PBS for 1 h. at room temperature.
   ** coating antigen concentration must be determined in advance for each batch. Normally, twice the minimum saturating concentration is used.
2. Wash three times with PBS-T.
3. Block wells of microtiter plate with 200 µl/well of 1% BSA in PBS for 1 h. at room temperature.
4. Wash three times with PBS-T.
5. Add 50 µl/well sample containing free PTX and 50 µl/well optimally diluted 73D3 anti-palytoxin murine monoclonal antibody (or anti-palytoxin rabbit antibody preparation), diluted in PBS-B. Incubate for 1 h. at room temperature.
6. Wash three times with PBS-T.
7. Add 100 µl/well Alkaline Phosphatase goat anti-mouse Ig conjugate (or alkaline phosphatase goat anti-rabbit IgG if anti-palytoxin rabbit antibody preparation is used), diluted to appropriate concentration with 1% BSA in PBS. Incubate 1 h. at room temperature.
8. Wash four times with TBS-T.
9. Add 200 µl/well 1 mg/ml pNPP diluted in alkaline phosphatase substrate buffer, pH 9.5.

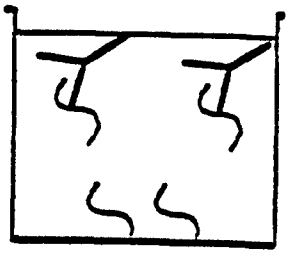
10. Read on dual wavelength absorbance mode (reference wavelength = 690 nm = filter 8 on Titertek; sample wavelength = 414 nm = filter 3 on Titertek).

Indirect CIEIA using 73D3 MAb and AP-Anti-Mouse Ig with PTX-M Coated Solid Phase



1. PTX-M-BSA coated on solid phase.

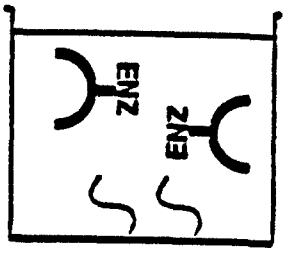
Incubate then wash.



2. Test sample +/- free PTX added.

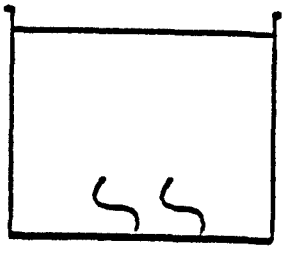
73D3 MAb at optimized dilution added.

Incubate then wash.



3. AP-Anti-Mouse Ig at optimized dilution added.

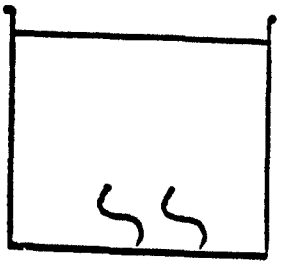
Incubate then wash.

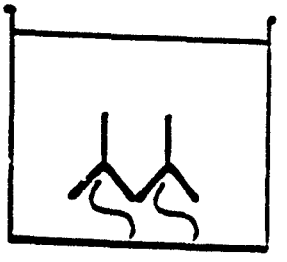


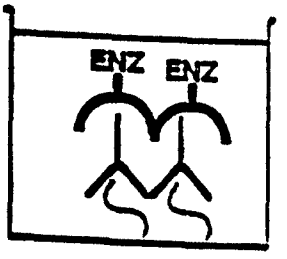
4. AP Substrate added.

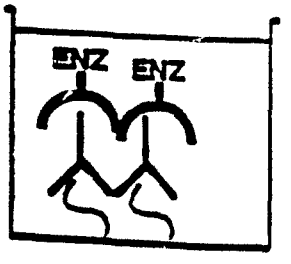
Incubate then read.

Positive









Negative

APPENDIX O

Indirect Sandwich ELISA for Palytoxin
March, 1990

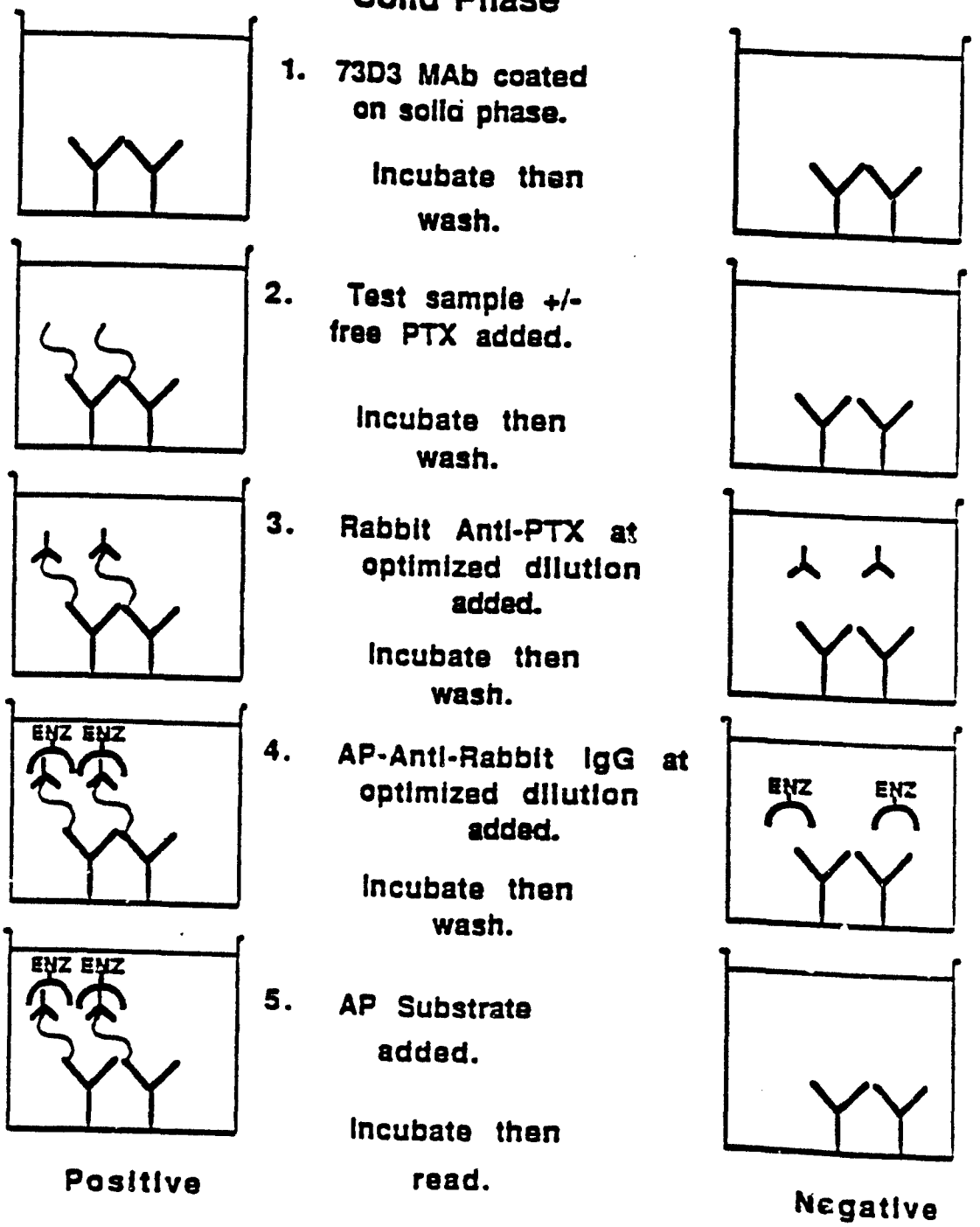
Materials:

1. Immulon 2 microtiter plates (Dynatech)
2. 40% saturated ammonium sulfate purified 73D3 monoclonal antibody
3. Rabbit anti-palytoxin Ig fraction (Rabbit 007, bleed 5, "R7B7")
4. 50 mM Sodium Phosphate, pH 7.0, 0.15 M NaCl (PBS)
5. 50 mM Sodium Phosphate, pH 7.0, 0.15 M NaCl, 0.5 mM $Na_2B_4O_7$ (PBS-B)
5. PBS containing 0.05% (v/v) Tween-20 (PBS-T)
6. 50 mM Tris HCl, pH 7.0, 0.15 M NaCl, 0.05% (v/v) Tween 20 (TBS-T)
7. Bovine Serum Albumin (BSA)
8. Alkaline Phosphatase goat anti-rabbit Ig conjugate
9. *p*-nitrophenylphosphate (pNPP) (Sigma 104, 5 mg tablets)
10. Alkaline Phosphatase substrate buffer:
    25 mM Trizma base, pH 9.5
    0.15 M NaCl
    5 mM $MgCl_2$
    0.02% (w/v) NaN_3

Method:

1. Coat Immulon 2 microtiter plate wells with 40% saturated ammonium sulfate purified 73D3 MAb, 100 µl/well (** µg/well in PBS, pH 7.0)
   ** coating antigen concentration must be determined in advance for each batch. Normally, twice the minimum saturating concentration is used.
2. Wash three times with PBS-T.
3. Block wells of microtiter plate with 200 µl/well of 1% BSA in PBS for 1 h. at room temperature.
4. Wash three times with PBS-T.
5. Add PTX standards, or test samples, 1% BSA in PBS-Borate, 100µl/well. Incubate 1 h. at room temperature.
6. Wash three times with PBS-T.
7. Add 100µl/well rabbit anti-PTX antibody (R7B7) 1/3000 in 1% BSA in PBS for 1 h. at room temperature.
8. Wash three times with PBS-T.
9. Add 100µl/well alkaline phosphatase goat anti-rabbit Ig conjugate diluted to manufacturers recommended dilution in 1% BSA in PBS. Incubate at room temperature for 1 h.
10. Wash four times with TBS-T.
11. Add 200 µl/well 1 mg/ml pNPP diluted in alkaline phosphatase substrate buffer, pH 9.5.
12. Read on dual wavelength absorbance mode (reference wavelength = 690 nm = filter 8 on Titertek; sample wavelength = 414 nm = filter 3 on Titertek).

Indirect Sandwich ELISA using Rabbit Anti-PTX and AP-Anti-Rabbit IgG with 73D3 MAb Coated Solid Phase



APPENDIX P

Direct CIEIA for Palytoxin using AP-73D3 Monoclonal Antibody
March, 1990

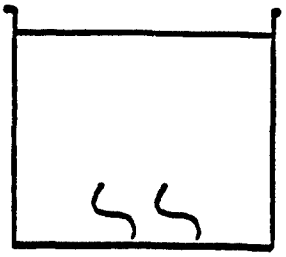
Materials:

1. Immulon 2 microtiter plates (Dynatech)
2. PTX-MCC-BSA coating antigen
3. Protein A-Sepharose purified 73D3 Monoclonal Antibody, conjugated to Alkaline Phosphatase (AP-73D3)
4. 50 mM Sodium Phosphate, pH 7.0, 0.15 M NaCl (PBS)
5. PBS containing 0.05% (v/v) Tween-20 (PBS-T)
6. 50 mM Sodium Phosphate, pH 7.0, 0.15 M NaCl, 0.5 mM $Na_2B_4O_7$ (PBS-B)
7. 50 mM Tris HCl, pH 7.0, 0.15 M NaCl, 0.05% (v/v) Tween 20 (TBS-T)
8. Bovine Serum Albumin (BSA)
9. *p*-nitrophenylphosphate (pNPP) (Sigma 104, 5 mg tablets)
10. Alkaline Phosphatase substrate buffer:
    25 mM Trizma base, pH 9.5
    0.15 M NaCl
    5 mM $MgCl_2$
    0.02% (w/v) NaN_3

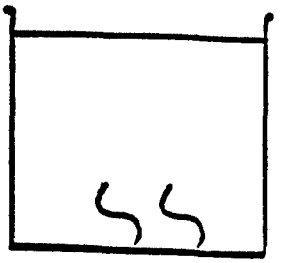
Method:

1. Coat Immulon 2 microtiter plates with PTX-MCC-BSA, 100 µl/well, at ** µg/ml in PBS for 1 h. at room temperature.
   ** coating antigen concentration must be determined in advance for each batch. Normally, twice the minimum saturating concentration is used.
2. Wash three times with PBS-T.
3. Block wells of microtiter plate with 200 µl/well of 1% BSA in PBS for 1 h. at room temperature.
4. Wash three times with PBS-T.
5. Add 50 µl/well PTX samples, titrated in PBS-B, and 50 µl/well optimally diluted AP-73D3 MAb. Incubate for 1 h. at room temperature.
6. Wash four times with TBS-T.
7. Add 200 µl/well 1 mg/ml pNPP diluted in alkaline phosphatase substrate buffer, pH 9.5.
8. Read on dual wavelength absorbance mode (reference wavelength = 690 nm = filter 8 on Titertek; sample wavelength = 414 nm = filter 3 on Titertek).

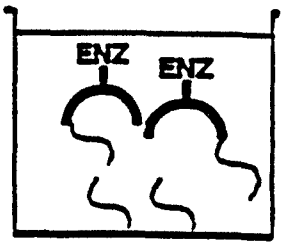
Direct CIEIA using AP-73D3 MAb with PTX-M Coated Solid Phase



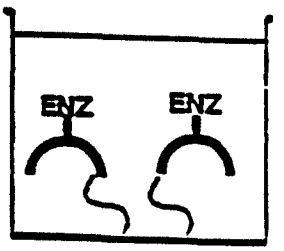
1. PTX-M-BSA coated on solid phase.



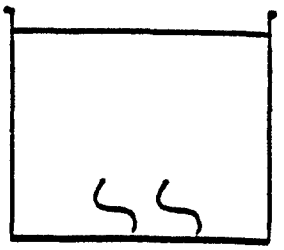
Incubate then wash.



2. Test sample +/- free PTX added.
AP-73D3 MAb at optimized dilution added.

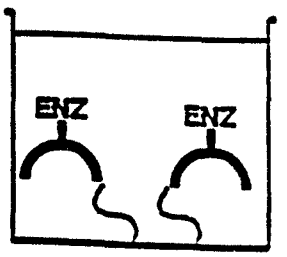


Incubate then wash.



3. AP Substrate added.

Incubate then read.



Positive

Negative

APPENDIX Q

Direct CIEIA for Palytoxin using Alkaline Phosphatase-Palytoxin Conjugate
March, 1990

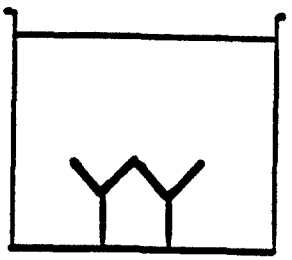
Materials:

1. Immulon 2 microtiter plates (Dynatech)
2. 40% saturated ammonium sulfate purified 73D3 monoclonal antibody
3. Alkaline Phosphatase-Palytoxin conjugate (AP-PTX)
4. 50 mM Sodium Phosphate, pH 7.0, 0.15 M NaCl (PBS)
5. 50 mM Sodium Phosphate, pH 7.0, 0.15 M NaCl, 0.5 mM $Na_2B_4O_7$ (PBS-B)
6. PBS containing 0.05% (v/v) Tween-20 (PBS-T)
7. 50 mM Tris HCl, pH 7.0, 0.15 M NaCl, 0.05% (v/v) Tween 20 (TBS-T)
8. Bovine Serum Albumin (BSA)
9. *p*-nitrophenylphosphate (pNPP) (Sigma 104, 5 mg tablets)
10. Alkaline Phosphatase substrate buffer:
    25 mM Trizma base, pH 9.5
    0.15 M NaCl
    5 mM $MgCl_2$
    0.02% (w/v) NaN_3

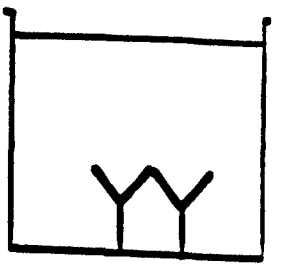
Method:

1. Coat Immulon 2 microtiter plate wells with 40% saturated ammonium sulfate purified 73D3 MAb, 100 µl/well (** µg/well in PBS, pH 7.0)
   ** coating antigen concentration must be determined in advance for each batch. Normally, twice the minimum saturating concentration is used.
2. Wash three times with PBS-T.
3. Block wells of microtiter plate with 200 µl/well of 1% BSA in PBS for 1 h. at room temperature.
4. Wash three times with PBS-T.
5. Add 50 µl/well PTX samples, titrated in PBS-B, and 50 µl/well optimally diluted AP-PTX. Incubate for 1 h. at room temperature.
6. Wash four times with TBS-T.
7. Add 200 µl/well 1 mg/ml pNPP diluted in alkaline phosphatase substrate buffer, pH 9.5.
8. Read on dual wavelength absorbance mode (reference wavelength = 690 nm = filter 8 on Titertek; sample wavelength = 414 nm = filter 3 on Titertek).

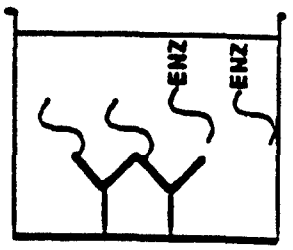
CIEIA using AP-PTX with 73D3 MAb Coated Solid Phase



1. 73D3 MAb coated on solid phase.

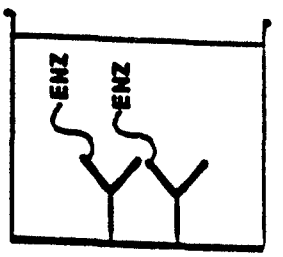


Incubate then wash.

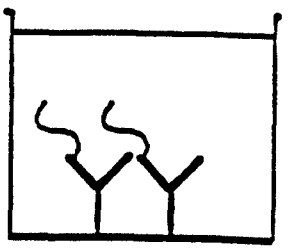


2. Test sample +/- free PTX added.

AP-PTX added at optimized dilution.

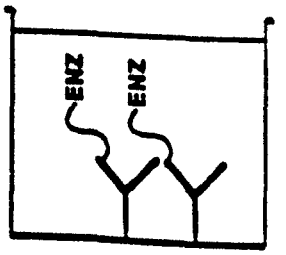


Incubate then wash.



3. AP Substrate added.

Incubate then read.



Positive

Negative

APPENDIX R

Direct Sandwich ELISA for Palytoxin

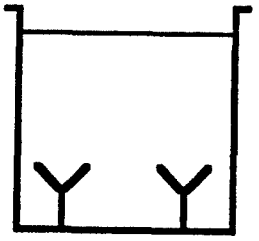
Materials:

1. Immulon 2 microtiter plates (Dynatech)
2. 40% saturated ammonium sulfate purified 73D3 monoclonal antibody
3. Alkaline phosphatase labelled rabbit anti-palytoxin conjugate (AP-R7B7)
4. 50 mM Sodium Phosphate, pH 7.0, 0.15 M NaCl (PBS)
5. 50 mM Sodium Phosphate, pH 7.0, 0.15 M NaCl, 0.5 mM $Na_2B_4O_7$ (PBS-B)
5. PBS containing 0.05% (v/v) Tween-20 (PBS-T)
6. 50 mM Tris HCl, pH 7.0, 0.15 M NaCl, 0.05% (v/v) Tween 20 (TBS-T)
7. Bovine Serum Albumin (BSA)
8. *p*-nitrophenylphosphate (pNPP) (Sigma 104, 5 mg tablets)
9. Alkaline Phosphatase substrate buffer:
   25 mM Trizma base, pH 9.5
   0.15 M NaCl
   5 mM $MgCl_2$
   0.02% (w/v) NaN_3

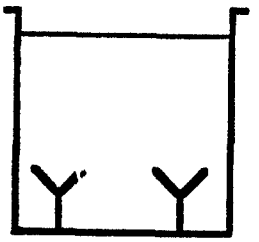
Methods:

1. Coat Immulon 2 microtiter plate wells with 40% saturated ammonium sulfate purified 73D3 MAb, 100 µl/well (** µg/well in PBS, pH 7.0)
   ** coating antigen concentration must be determined in advance for each batch. Normally, twice the minimum saturating concentration is used.
2. Wash three times with PBS-T.
3. Block wells of microtiter plate with 200 µl/well of 1% BSA in PBS for 1 h. at room temperature.
4. Wash three times with PBS-T.
5. Add PTX standards, or test samples, 1% BSA in PBS-Borate, 100µl/well. Incubate 1 h. at room temperature.
6. Wash three times with PBS-T.
7. Add 100µl/well rabbit AP-R7B7 optimally diluted in 1% BSA in PBS. Incubate 1 h. at room temperature.
8. Wash three times with PBS-T.
9. Wash four times with TBS-T.
10. Add 200 µl/well 1 mg/ml pNPP diluted in alkaline phosphatase substrate buffer, pH 9.5.
11. Read on dual wavelength absorbance mode (reference wavelength = 690 nm = filter 8 on Titertek; sample wavelength = 414 nm = filter 3 on Titertek).

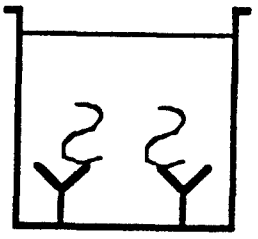
Direct Sandwich Enzyme Immunoassay for Detection of Palytoxin



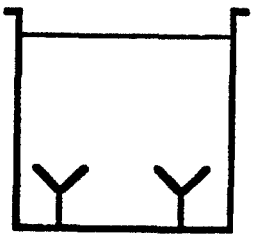
1.'capture' antibody coated on solid phase



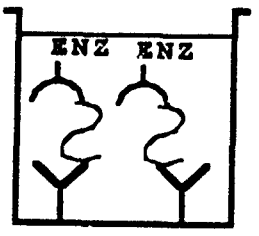
Incubate then wash



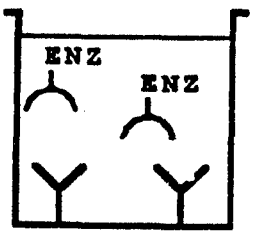
2. Test sample +/- free palytoxin added



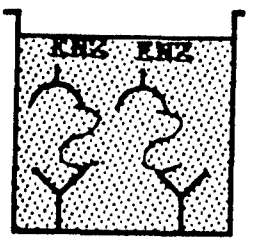
Incubate then wash



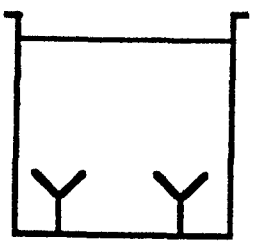
3. Enzyme-labelled anti-palytoxin at optimized dilution added.



Incubate then wash



4. Enzyme substrate added.



Positive

Incubate then read optical density

Negative

APPENDIX S

In Vitro ^{14}C-Leucine Incorporation Cytotoxicity Assay for Palytoxin

NOTE: Steps 1-7 require aseptic technique

1. Culture murine T-cell leukemia EL-4 cells in Dulbecco's minimal essential medium supplemented with 10% (v/v) calf serum, 2 mM L-glutamine, and 1 mM sodium pyruvate (suppl. MEM).
2. Harvest EL-4 cells by centrifugation at 200 x g for 7 minutes.
3. Dilute purified palytoxin standard preparation to 50 - 0.5 pg/ml in suppl. MEM. Dilute palytoxin "unknowns" that require testing.
4. To each well of a 96-well sterile tissue culture tray, add 50 µl of the EL-4 suspension. Then add 50 µl of diluted palytoxin standards, diluted palytoxin "unknowns" or medium controls to appropriate wells.
5. Incubate for 18 hours in 5% CO_2/95% humidified air at 37 °C.
6. Add 50 µl suppl. MEM containing 0.1 µCi ^{14}C-Leucine to each well.
7. Incubate for 2 hours in 5% CO_2/95% humidified air at 37 °C.
8. Harvest contents of wells onto glass fiber filters using MiniMash™ unit (MA Bioproducts). Chase with 50 ml water, then at least 50 ml 95% ethanol.
9. Dry filters at 50 °C under vacuum for at least one hour.
10. Place discs in scintillation cocktail and count radioactivity.
11. Plot results relative to plain medium controls.

J.HEWETSON, ET AL. ABSTRACT

Reference: Hewtson, J.F., G. Bignami and D.C. Vann. 1989. *Faseb Journal* 3: A1191.

IN-VITRO AND *IN-VIVO* PROTECTION BY MONOCLONAL ANTIBODY AGAINST PALYTOXIN EXPOSURE. J.F. Hewetson, G. Bignami, and D.C. Vann (SPON: R.W. Wannemacher, Jr.). U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD 21701-5011 and Hawaii Biotechnology Group, Aiea, HI 96701.

Palytoxin, one of the most toxic, non-proteinaceous compounds known, has several biological properties which may directly or indirectly contribute to its toxicity. A monoclonal antibody raised against palytoxin by conjugation of the molecule to keyhole limpet hemocyanin inhibited the cytotoxicity of palytoxin for EL-4 murine lymphoma cells in a [^{14}C]leucine incorporation assay. Palytoxin was ≥95% cytotoxic at 25 pg/ml and the monoclonal antibody gave 50% inhibition at 2.5 ug/ml (molar ratio = 1:3,500). A mouse LD_{99} dose of palytoxin premixed with antibody at a molar ratio of 1:300 and injected i.v. delayed death by 8 hours. The same mixture injected i.p. did not delay death. When antibody was injected i.v., followed immediately by an i.p. challenge with palytoxin, death was delayed by more than 24 hours. These results suggest that monoclonal antibody neutralized at least some of palytoxin's toxic biological properties, and may provide a tool for additional studies on mechanism of action.

APPENDIX U

Summary of Raw Data from Testing Crude *Palythoa* Extracts for Parallelism using CIEIA.

This table presents the raw data values for calculated palytoxin content of a seies of crude extracts of *Palythoa tuberculosa* which were subjected to serial dilutions and then tested in the standard CIEIA format.

The following information is intended to aid in understanding how the table is arranged.

Column or Row Headings	Explanation
1. EXT. NO--Column heading	In this series of experiments, extracts were numbered 1-10.
2. DILUTION--Column heading	The crude extracts were subjected to serial dilution in standard PBS-BSA buffer.
3. DATES--Column heading	The extracts were tested seven times on the dates indicated.
4. MEAN--Column heading	Italicized values in this column are the means of successive determinations done at each of the indicated dilutions.
5. ST. DEV.--Column heading	Italicized values in this column are the standard deviations of successive determinations done at each of the indicated dilutions.
6. % CV--Column heading	Italicized values in this column are the percent coefficients of variation of successive determinations done at each of the indicated dilutions.
7. MEAN--Row heading	Italicized values in these rows are the mean of all of the usable data for the indicated day.
8. ST. DEV.--Row heading	Italicized values in these rows are the standard deviations of all of the usable data for the indicated day.
9. DATA VALUES	Standard typeface numbers are calculated palytoxin concentration in µg/ml. They were obtained from the mean of triplicate CIEIA O.D. readings interpolated from each day's standard palytoxin inhibition curve. The entry *** indicates unusable data points.

For each extract there are seven boldface numbers which are given in the following array:

	A	
B	C	D
E	F	G

The definition of each of these entries is as follows:

A Number of observations providing useful data.
B Mean of all the useful data.
C Standard deviation of all the useful data.
D Percent coefficient of variation of all the useful data.
E Standard error of all the useful data.
F Mean minus one standard error (A - E).
G Mean plus one standard error (A + E).

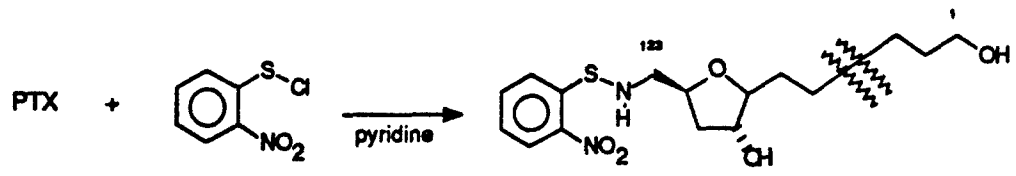
EXT. NO	DILUTION	4/7/88	4/8/88	4/12/88	4/13/88	4/14/88	4/28/88	4/29/88	MEAN	ST. DEV.	% CV
1	1/100	4.4	2.6	2.8	2.7	2.5	2.9	1.9	2.83	0.77	27
	1/400	3.8	2.2	3.2	3.2	3.4	3.4	2.3	3.07	0.60	19
	1/1600	4.2	***	***	4.5	5.6	6.4	3.2	4.78	1.25	26
	1/6400	7.7	***	***	***	***	***	2.1		3.96	#####
	MEAN	5.03	2.40	3.00	3.47	3.83	4.23	2.38		21	
	ST. DEV.	1.80	0.28	0.28	0.93	1.59	1.89	0.57	3.57	1.48	42
									0.32	3.25	3.90
2	1/100	3	4	2.5	3	4.1	5.4	2.1	3.44	1.13	33
	1/400	3.4	4.8	3.2	4.4	5.6	5	3.1	4.21	0.99	23
	1/1600	3.4	4.6	***	5.6	8	6.7	3.9	5.37	1.75	33
	1/6400	4.1	***	***	***	***	***	***	4.10	#####	#####
	MEAN	3.48	4.47	2.85	4.33	5.90	5.70	3.03		21	
	ST. DEV.	0.46	0.42	0.49	1.30	1.97	0.89	0.90	4.28	1.43	33
									0.31	3.97	4.59
3	1/100	***	***	***	**	***	***	14.7	14.70	#####	#####
	1/400	26.4	16	15.4	10.4	16	15.9	12.1	16.03	5.08	32
	1/1600	28.8	22.4	22.4	13.1	16.8	19.5	14.9	19.70	5.36	27
	1/6400	22.4	***	***	***	16	26.5	***	21.63	5.29	24
	MEAN	25.87	19.20	18.90	11.75	16.27	20.63	13.90		18	
	ST. DEV.	3.23	4.53	4.95	1.91	0.46	5.39	1.56	18.32	5.31	29
									1.25	17.06	19.57
4	1/100	5.6	6	3.8	***	4	3.2	3.6	4.37	1.15	26
	1/400	5.6	7.2	5.2	2.8	6	4.8	4.9	5.21	1.34	26
	1/1600	***	6.4	8.3	4.4	5.6	7.5	5.9	6.35	1.39	22
	1/6400	21.1	***	***	***	***	***	***		#####	#####
	MEAN	10.77	6.53	5.77	3.60	5.20	5.17	4.80		20	
	ST. DEV.	8.95	0.61	2.30	1.13	1.06	2.17	1.15	6.10	3.81	63
									0.85	5.24	6.95

5	1/100	***	***	***	***	***	***	***	#####	#####	#####	
	1/400	28.4	24	9.6	20	24	30.4	20.9	22.47	6.79	30	
	1/1600	25.6	22.4	24	30.4	24	27.6	23.8	25.40	2.75	11	
	1/6400	30.7	16	35.2	20.5	28.1	40	22.4	27.56	8.50	31	
	MEAN	28.23	20.80	22.93	23.63	25.37	32.67	22.37		21		
	ST. DEV.	2.55	4.23	12.83	5.87	2.37	6.50	1.45	25.14	6.51	26	
									1.42	23.72	26.56	
6	1/100	1.6	1.7	1.6	1.4	1.9	1.4	1.1	1.53	0.26	17	
	1/400	1.9	1.8	3.2	1.5	2.2	2.6	1.6	2.11	0.61	29	
	1/1600	3	4.3	5.7	***	5.1	6.5	2.3	4.48	1.61	36	
	1/6400	8.9	***	***	***	***	***	***		#####	#####	
	MEAN	3.85	2.60	3.50	1.45	3.07	3.50	1.67		21		
	ST. DEV.	3.42	1.47	2.07	0.07	1.77	2.67	0.60	2.92	2.05	70	
									0.45	2.47	3.37	
7	1/100	0.72	0.52	1.4	0.66	1.1	0.72	0.44	0.79	0.34	43	
	1/400	1.2	***	2	0.96	3	1.6	0.65	1.57	0.85	54	
	1/1600	2.5	***	7.2	***	6.6	5.1	1.3	4.54	2.56	56	
	1/6400	***	***	***	***	***	17.6	***		#####	#####	
	MEAN	1.47	0.52	3.53	0.81	3.57	6.26	0.80		19		
	ST. DEV.	0.92	#DIV/0!	3.19	0.21	2.79	7.80	0.45	2.91	4.09	141	
									0.94	1.97	3.85	
8	1/100	6.6	***	5.4	5	9	4.1	3.4	5.58	2.00	36	
	1/400	7.6	10.4	9.2	7.6	12	4.7	4.1	7.94	2.88	36	
	1/1600	9.6	13.6	22.4	10.8	***	8.3	6.6	11.88	5.67	48	
	1/6400	22.4	30.7	41.6	28.9	***	***	***	30.90	7.97	26	
	MEAN	11.55	18.23	19.65	13.08	10.50	5.70	4.70		23		
	ST. DEV.	7.34	10.91	16.35	10.81	2.12	2.27	1.68	12.35	10.02	81	
									2.09	10.26	14.44	

9	1/100	***	***	17.6	5	***	***	9.5	10.70	6.39	60	
	1/400	22	13.6	25.6	6	14	14.6	8.7	14.93	6.89	46	
	1/1600	24	15.2	35.2	33.2	16	17.9	13.5	22.14	8.90	40	
	1/6400	23	***	***	***	***	34.5	18	25.17	8.46	34	
	MEAN	23.00	14.40	26.13	14.73	15.00	22.33	12.43		20		
	ST. DEV.	1.00	1.13	8.81	16.00	1.41	10.67	4.27	18.36	8.83	48	
									1.98	16.38	20.33	
10	1/100	26.4	***	***	***	***	16.8	13	18.73	6.91	37	
	1/400	30.4	28	24	28	22	18.5	11.2	23.16	6.66	29	
	1/1600	35.8	35.2	41.6	35.2	28.8	34.1	13.9	32.09	8.84	28	
	1/6400	***	38.4	***	67.2	47	***	23.6	44.05	18.21	41	
	MEAN	30.87	33.87	32.80	43.47	32.60	23.13	15.43		21		
	ST. DEV.	4.72	5.33	12.45	20.87	12.93	9.54	5.56	29.48	12.94	44	
									2.82	26.66	32.30	

APPENDIX V

Synthesis of *S*-(2'nitrophenyl)palytoxin sulfenamide (NPS-PTX)



NPS-PTX

o-Nitrophenylsulfenyl chloride (2.8 mg,15 µmol) was added to a solution of palytoxin (8.1 mg, 3.0 µmol) in pyridine (1.2 ml). After being stirred for 12 hours at room temperature, the reaction mixture was diluted with water, filtered and concentrated. The resultant residue was purified by preparative HPTLC (E. Merck NH_2 F_{254}, #15647· :8:7 pyridine-H_2O-*n*-AmOH) to give NPS-PTX in 76% yield.

The UV spectrum of the product has the usual PTX absoptions at 233 and 263 nm and a new peak at 349 nm corresponding to the NPS group. The NMR spectrum has not yet been assigned, although peaks corresponding to the protons of the aromatic reing are clearly visible.

APPENDIX W

Palytoxin
Storage and Preparation for Use

1. **Storage:**
   - Store bulk sample dry at -20 °C under argon or nitrogen atmosphere

2. **For use:**
   - Dissolve contents of flask in 1.0 ml water.
   - Withdraw the desired aliquot (e.g. 20 µl).
   - Dilute to 1 mg/ml in 50% aqueous ethanol.
   - Store this working stock tightly capped at -20 °C.
   - Return main stock to dryness (see # 4 below).

3. **Working stock:**
   - On the day of use, characterize and quantitate by UV spectroscopy
   - Dilute an aliquot 1:50 in water to yield ~0.02 mg/ml.
   - Scan OD of the diluted aliquot from 190 to 300 nm.
   - Calculate palytoxin concentration using ε_{263} = 23,600 (Ref: Moore, 1985).
   - (OD_{263} of the diluted aliquot should be ~0.15–0.20)
   - What to look for::
     - Refer to attached UV absorption spectrum.
     - Peaks at 233 and 263 nm should be in a ratio of ~ 1.6:1.
     - Absorption minimum at 200-210 nm should be about equal to or less than the absorption minimum at 250 nm.
     - Excessive increases over time in end absorbance at wavelengths below 200 nm may indicate degradation.

4. **Return of main stock to dryness**
   - Freeze contents of flask in dry ice/acetone bath.
   - Maintain frozen at -10 to -20 °C using NaCl/ice bath.
   - Lyophilize using a pump capable of pulling less than 0.006 ATM. (4.56 mm Hg).
   - This procedure may take up to three days
   - When dry, release vacuum, cap under argon and store at -20 °C.

5. **Reference:** Moore, R.E., IN *Progress in the Chemistry of Organic Natural Products*, vol. 48, Herz, W.; Grisebach, H.; Kirby, G.W.; Tamm, Ch. Springer-Verlag: New York, 1985, pp. 81-202.

DETERMINATION OF PALYTOXIN CONTENT

CALCULATIONS:

PALYTOXIN
PURIFIED 1/15/88 54 mg. m.w.- 2679
HIGHLY TOXIC COMPOUND
HAWAII BIOTECHNOLOGY GROUP, INC

DILUTION FACTOR (DF) = 1/0.00019

OD_{263} = 0.1792

$$\text{CONCENTRATION (MG/ML)} = OD_{263} \times \frac{\text{M.W.}}{\epsilon_{263}} \times \text{DF}$$

$$= 0.1792 \times \frac{2679 \text{ MG/M MOL}}{23{,}600 \text{ ML/M MOL}} \times 1/0.00019$$

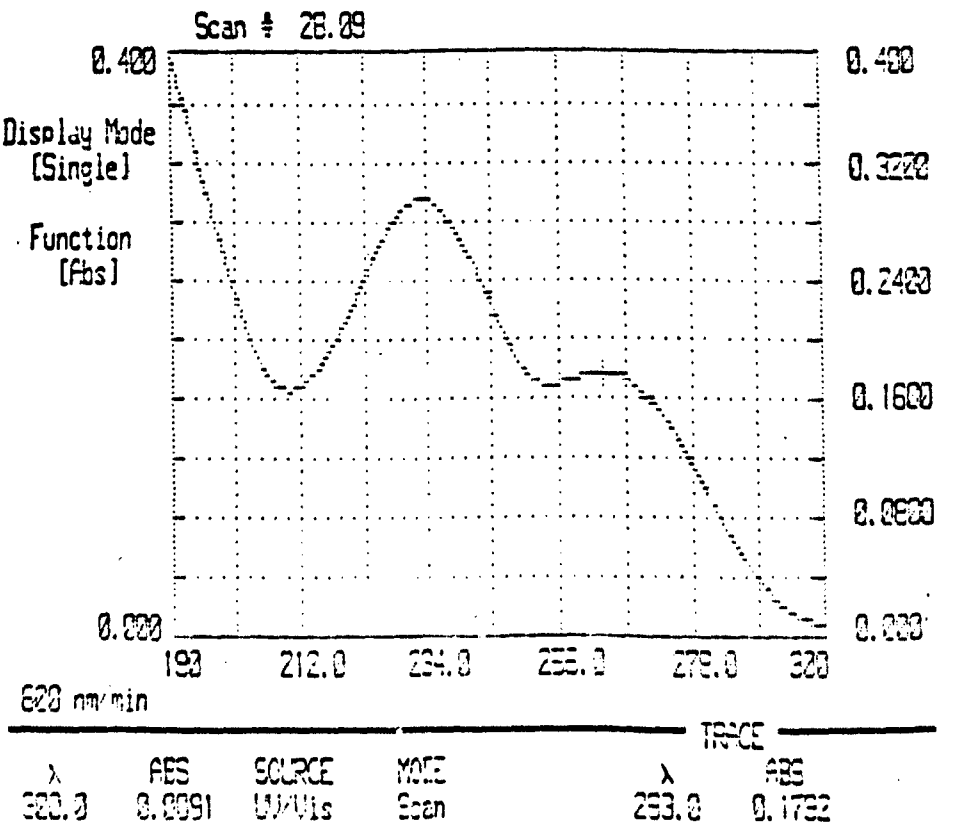
= 108 MG/ML

VOLUME USED = 0.5 ML

AMOUNT PALYTOXIN = 54 MG

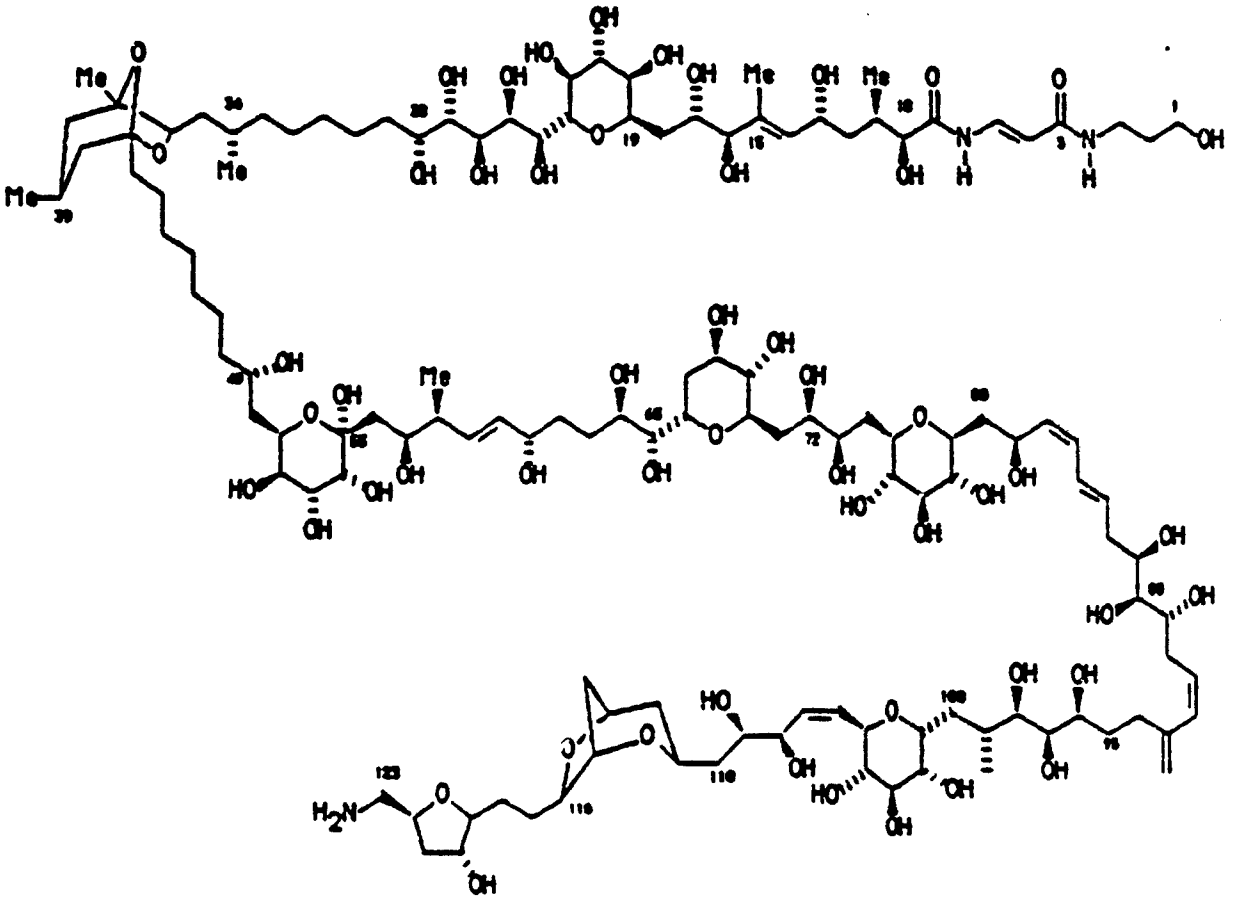
DATE: 1/14/88

SIGNATURE:_____



HAWAII BIOTECHNOLOGY GROUP, INC.

PALYTOXIN
Data Sheet



M.W. 2678.5
Source: *Palythoa tuberculosa* (9 vials @ 10 mg ea.)
Palythoa toxica (1 vial @ 10 mg)
Packaged March 14, 1989

Extremely potent toxin, skin irritant and tumor promotor: Use proper protective gear to prevent ingestion or contact with skin or eyes. In case of exposure, immediately wash affected area with copius amounts of soap and water. Transocular absorbtion can cause severe eye damage; in case of eye contact, flush eyes thoroughly with water and seek medical attention.

Spectral Data: UV spectra (H_2O) shown on next page.

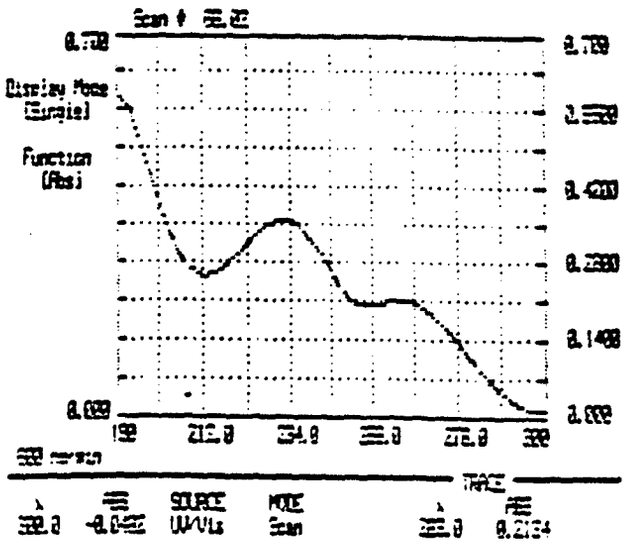
Purity: ≥95%

- HPLC: Both batches give a single peak with a slight shoulder. The shoulder peak is slightly smaller for the material isolated from *P. toxica* compared with the material from *P. tuberculosa* (4.6 X 250 mm Zorbax ODS column, 1:1 AcCN/0.05 N HOAc, UV 263 nm).

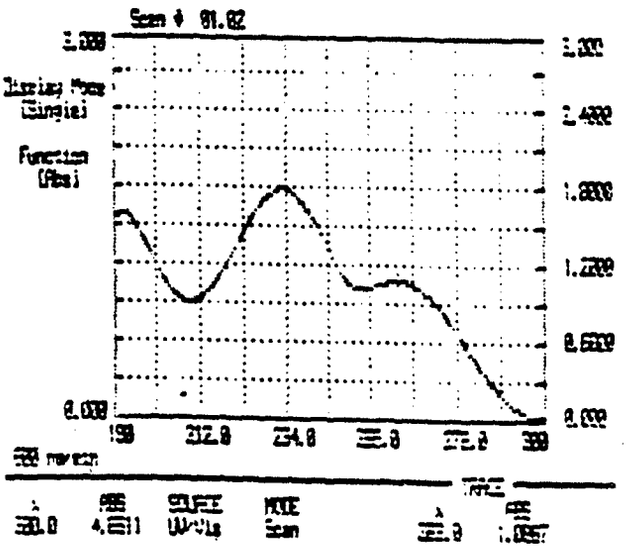
- UV: Both spectra are within the acceptable range.
  P. toxica $Abs_{233}/Abs_{263} = 1.65$
  P. tuberculosa $Abs_{233}/Abs_{263} = 1.70$

Solubility: Soluble in water, pyridine, DMSO; insoluble in most common organic solvents. Hygroscopic.

Storage: Store neat at -20° C under inert atmosphere (N_2 or Ar). Material was lyophilized and sealed under Ar.



from *P. tuberculosa*

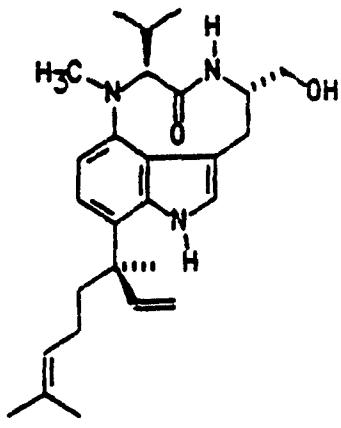


from *P. toxica*

LYNGBYATOXIN A DATA SHEETS

HAWAII BIOTECHNOLOGY GROUP, INC.

LYNGBYATOXIN A
Data Sheet



M.W. 437 Source: *Lyngbya majuscula*
Lot # 4-175-1 Purified March 16, 1989
$[\alpha]_D$ -170° (*c* 1.8, $CHCl_3$)

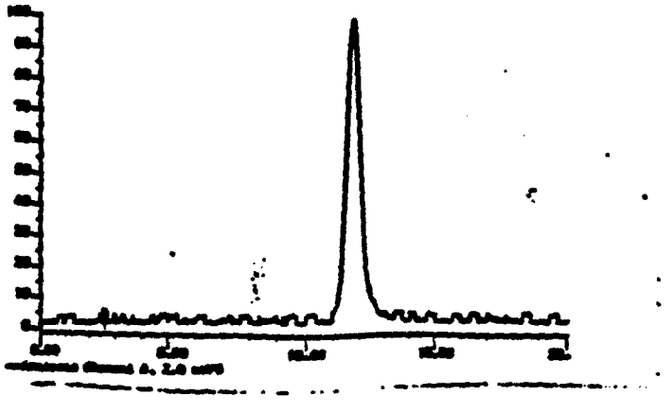
Extremely potent skin irritant and tumor promotor: Use proper protective gear to prevent contact with skin. In case of exposure, immediately wash affected area with copius amounts of soap and water.

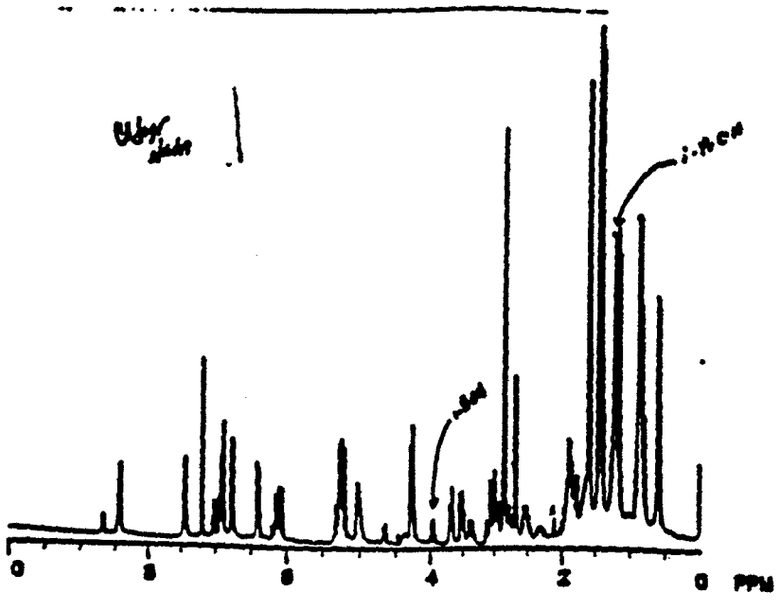
Purity: >95%; single spot by TLC (85:10:5 hexane/chloroform/isopropanol; silica gel); , single peak by HPLC (same solvent system and stationary phase as TLC, 240 nm, shown on next page); a trace of isopropanol is visible in 300 MHz NMR spectrum, this was removed by pumping an additional 12 h at 0.25 torr; doubling of some NMR signals, due to slow interconversion of conformers, is observed; NMR, UV and optical rotation match reported data (Cardellina, J.H., Ph.D. Thesis, University of Hawaii, 1979).

Spectral Data: NMR ($CDCl_3$) and UV (CH_3OH) shown on next page.

Solubility: practically insoluble in water; insoluble in hexane; soluble in CH_3OH, CH_2Cl_2, $CHCl_3$, acetone, DMSO.

Storage: Store neat at 0° C under inert atmosphere (N_2 or Ar). Slight decomposition observed after 48 hours at ambient temperature. **Hygroscopic.** Vials were packed under Ar.

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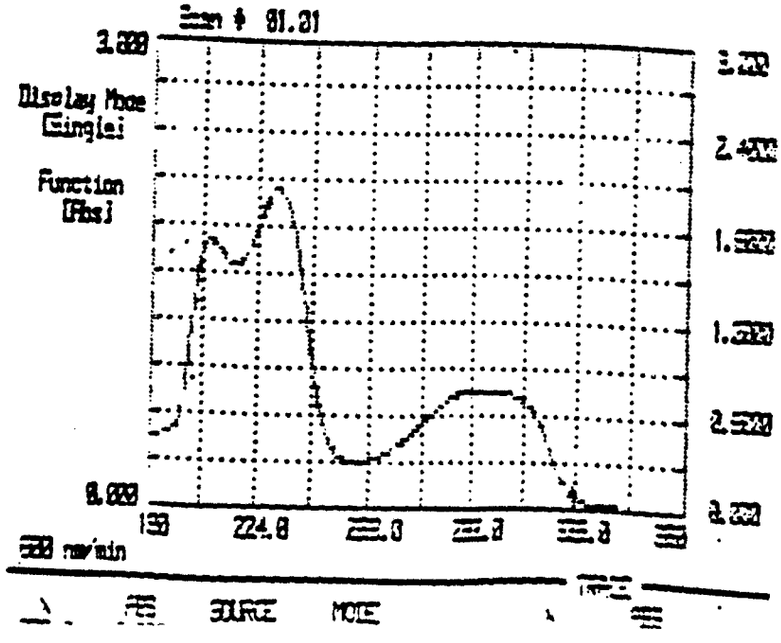
8
6
4
2
0
PPM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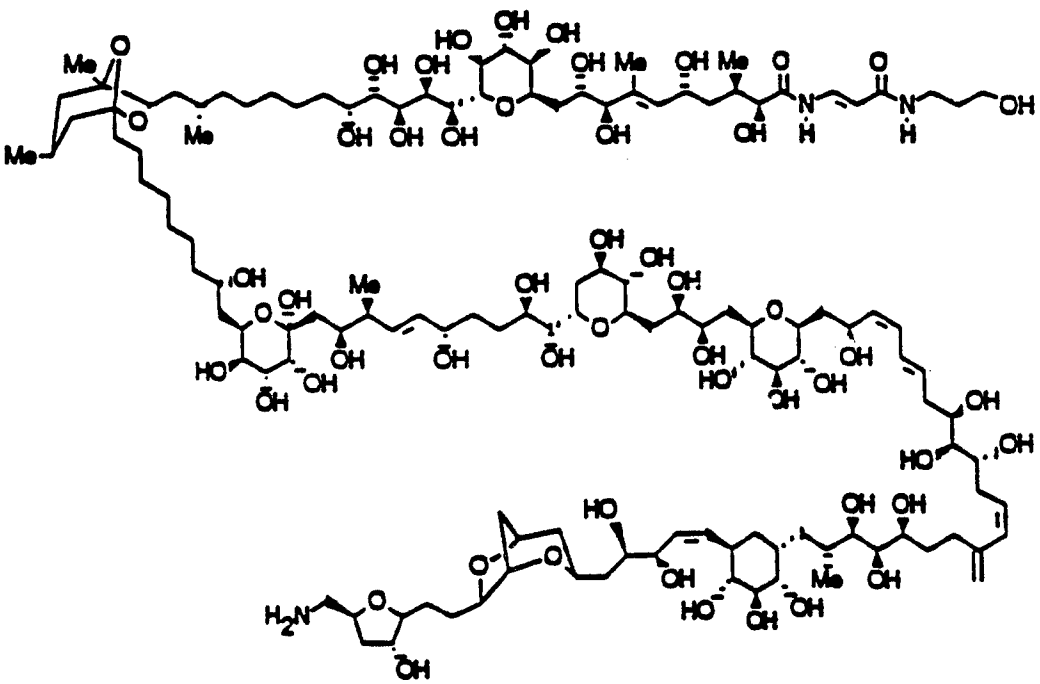
HAWAII BIOTECHNOLOGY GROUP, INC.

PALYTOXIN
Data Sheet



M.W. 2678.5
Source: *Palythoa tuberculosa* (7 vials @ 10 mg ea.)
Packaged January 31, 1990

Shipment Contents: This shipment contains 2 vials from batch 29-39-70 and 5 vials from batch 29-160. UV and NMR data indicate that 29-39-70 is of slightly better purity.

Extremely potent toxin, skin irritant and tumor promotor: Use proper protective gear to prevent ingestion or contact with skin or eyes. In case of exposure, immediately wash affected area with copius amounts of soap and water. Transocular absorbtion can cause severe eye damage; in case of eye contact, flush eyes thoroughly with water and seek medical attention.

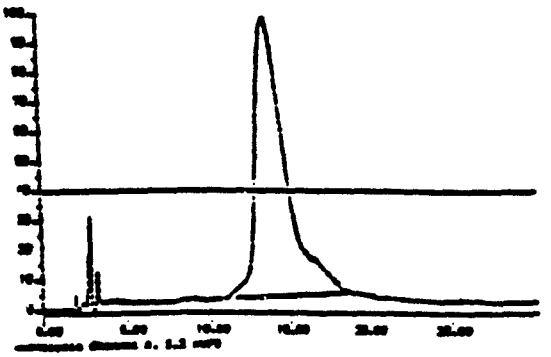
Solubility: Soluble in water, pyridine, DMSO; insoluble in most common organic solvents. Hygroscopic.

Storage: Store neat at -20° C under inert atmosphere (N_2 or Ar). Material was lyophilized and sealed under Ar.

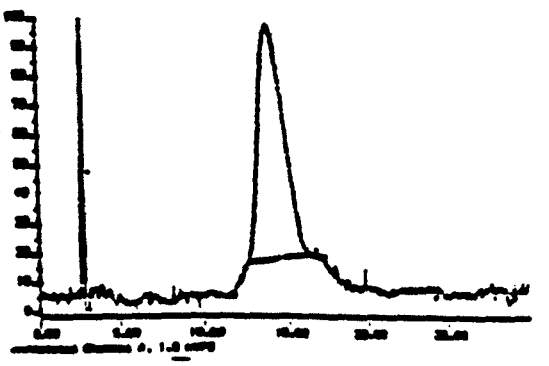
Spectral Data: UV spectra (H_2O) shown on next page.

Purity: ≥95%

- **HPLC:** Chromatograms shown below. (4.6 X 250 mm Zorbax ODS column, 40% AcCN/0.05 N HOAc, UV 263 nm).



29-39-70

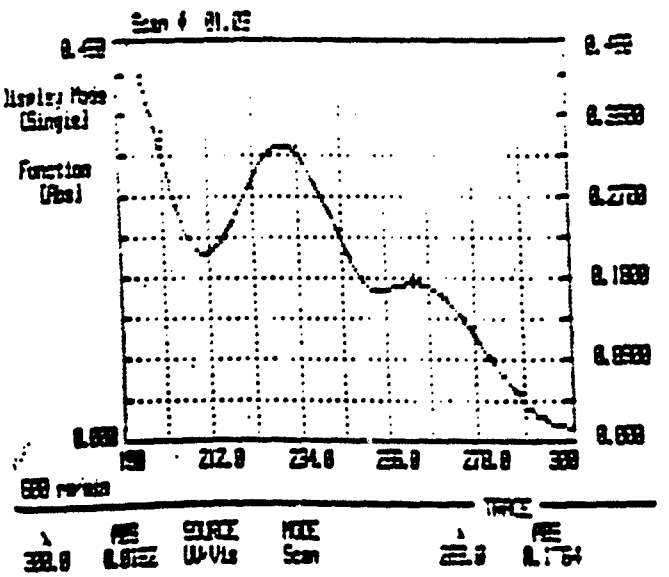


29-160

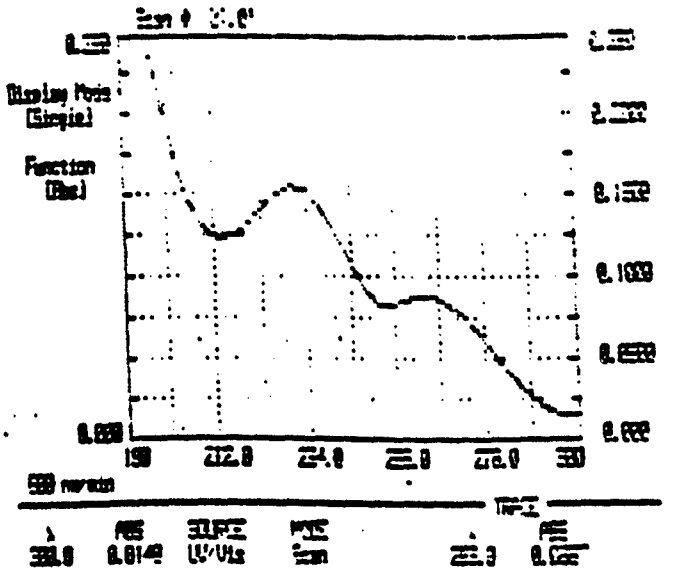
- **UV:** Both batches are within the acceptable range.

29-39-70; $Abs_{233}/Abs_{263} = 1.79$

29-160; $Abs_{233}/Abs_{263} = 1.75$



29-39-70



29-160

APPENDIX BB

HBG Technical Manual for Isolation, Purification, and Characterization of Palytoxin

With this final report, we have included a copy of our in-house manual for the training of isolation technicians. This has been revised each time there was a personnel change in this position and again at the conclusion of the project.

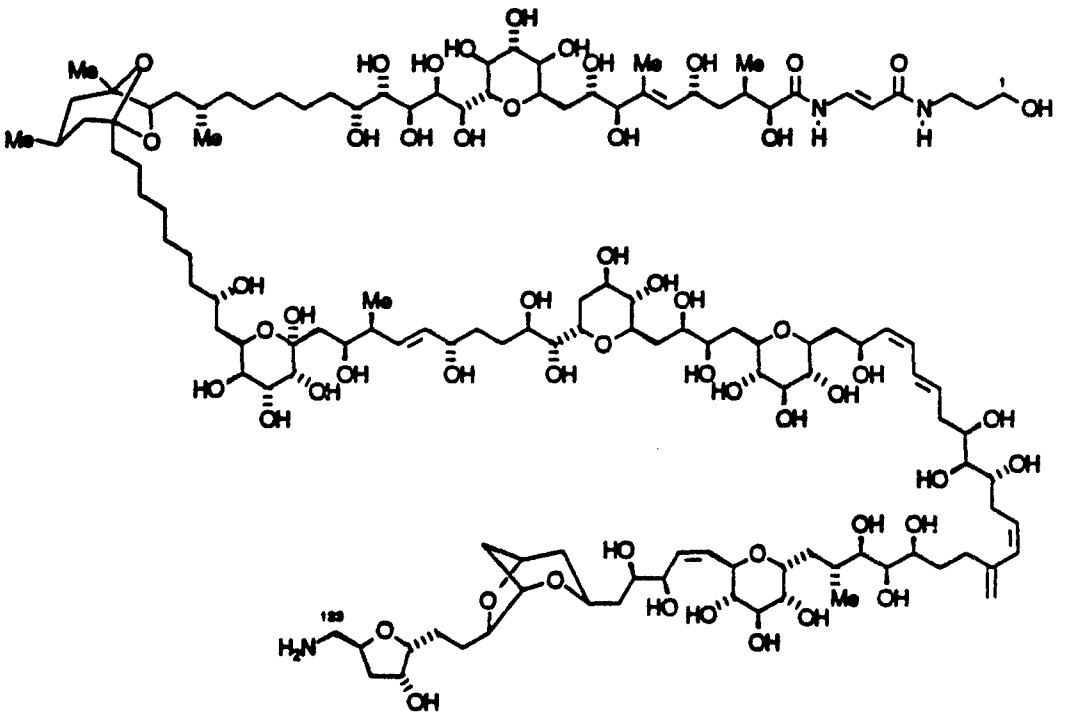
PALYTOXIN
ISOLATION AND PURIFICATION
PROCEDURES

Third Revision
March 25, 1991

Hawaii Biotechnology Group, Inc.
99-193 Aiea Heights Drive
Aiea, Hawaii 96701

PALYTOXIN ISOLATION

This manual includes detailed instructions for each procedure involved in the isolation of palytoxin from Palythoa tuberculosa as well as procedures for the preparation of all chromatography adsorbents. You must thoroughly familiarize yourself with the written procedures before attempting to perform these operations in the absence of your supervisor.



Palytoxin
M.W. 2678.5

SAFETY INFORMATION

Palytoxin is **extremely poisonous**. Toxin isolation work should never be performed while you are alone in the laboratory. Wear proper safety gear at all points in the isolation from start to end. That includes your lab jacket, safety glasses and heavy rubber gloves. If any of the juice splashes into your eyes, nose or mouth or if you cut yourself with toxin-contaminated equipment, **immediately** flush the affected area with copious amounts of water and notify other lab personnel to summon emergency medical aid. Contaminated clothing, benchtops, and equipment can be decontaminated by washing with a 10% aqueous bleach solution. For further information refer to the HBG Toxin S.O.P. which is available from the safety officer.

I. PREPARATION OF CHROMATOGRAPHY RESINS

A. AMBERLITE XAD-2: Sigma, non-ionic polymeric adsorbent; porosity volume ~42% (dry basis); surface area = 330 m^2/g; average pore diameter - 90Å; mesh size = 20-60.

A.1. Cleaning of New XAD-2 Resin

A.1.1. Pour new XAD-2 resin into a beaker and add 2x volume of deionized water.

A.1.2. Gently stir the mixture. Do not crush the beads against the walls of the beaker.

A.1.3. Allow the resin to settle. Fine resins will float on top of the water.

A.1.4. Decant the fine resin particles with the turbid water, retain the remainder of the resin in the beaker.

A.1.5. Repeat this process 5x or until water layer is clear and all the fines have been decanted.

A.1.6. Suspend the resin in acetone 2x volume, stir and allow to settle. Decant and repeat the process 3x.

A.1.7. Replace the acetone with methanol, stir and allow to settle. Decant and repeat process 3x.

A.1.8. Transfer the resin into a Soxhlet apparatus and wash overnight with methanol.

A.1.9. Replace the methanol in the Soxhlet apparatus with acetone, wash the resin overnight.

A.1.10. Transfer the resin from Soxhlet into a beaker and wash repeatedly with deionized water until no acetone or methanol odor can be detected.

A.1.11. The resin is then ready for use.

A.2. Regeneration of Used XAD-2 Resin

A.2.1. Carefully transfer used resin from the column into the Soxhlet apparatus.

A.2.2. Repeat steps A.1.8 through A.1.11. (NOTE: Methanol wash should continue until solvent is colorless. This may take 12-48 hours.

NOTE: XAD-2 RESIN MAY BE REGENERATED UP TO 6X AFTER WHICH ITS ADSORBING EFFICIENCY IS DECREASED.

B. DEAE SEPHADEX A-25: Sigma; anion exchanger; bead size: 40-120 µ; capacity: 3.5 0.5 meq/g; bed volume: 5-9 ml/g in tris HCl buffer, pH 8.3; ionic strength 0.05

B.1. Suspend DEAE Sephadex A-25 in deionized water at 35 °C. Stir gently and allow resin to settle.

B.2. Decant the fine gels and repeat the process until there are no more suspended fines.

B.3. Wash gel in a Büchner funnel with 70% EtOH (4x gel volume).

B.4. Wash with deionized water until the EtOH odor is no longer detectable.

B.5. Transfer gel back into a beaker and reswell it in water at 35 °C for 1 hour.

B.6. Wash gel in a Büchner funnel with 0.5 N NaOH (4x gel volume), repeat with water.

B.7. Wash gel with 0.1M H_3PO_4, repeat with water.

B.8. Wash with 0.5 N phosphate buffer, pH 7.0.

B.9. Wash with 0.2 N phosphate buffer, pH 7.0.

B.10. Finally wash with 0.02 N phosphate buffer, pH 7.0.

B.11. Store prepared DEAE gel in 0.02 N phosphate buffer, pH 7.0 with 0.4% azide at 4 °C until needed.

NOTE: ONCE STEP B.6 HAS BEEN STARTED, CLEANING PROCEDURE HAS TO BE FINISHED. STRONG ACID OR BASE CAN DESTROY THE DEAE GEL.

C. CM-SEPHADEX C-25: Sigma; cation exchanger; bead size: 40-120µ; capacity: 4.5-0.5 meq/g; bed volume: 6.1 ml/g in sodium phosphate buffer, pH 6; ionic strength 0.06

C.1. Suspend CM Sephadex C-25 from the botle in deionized water at 35 °C and allow resin to settle.

C.2. Decant the fine resin particles and repeat the process until there are no more suspended fines.

C.3. Wash the gel in Büchner funnel with deionized water (4x gel volume).

C.4. Wash with 30% EtOH (4x gel volume).

C.5. Wash with 50% EtOH (4x gel volume), then with water until no EtOH odor can be detected.

C.6. Transfer gel back into the beaker and reswell with deionized water at 35 °C for 1 hour.

C.7. Wash gel in Büchner funnel with 0.5 M NaH_2PO_4, pH 4.5 (4x gel volume).

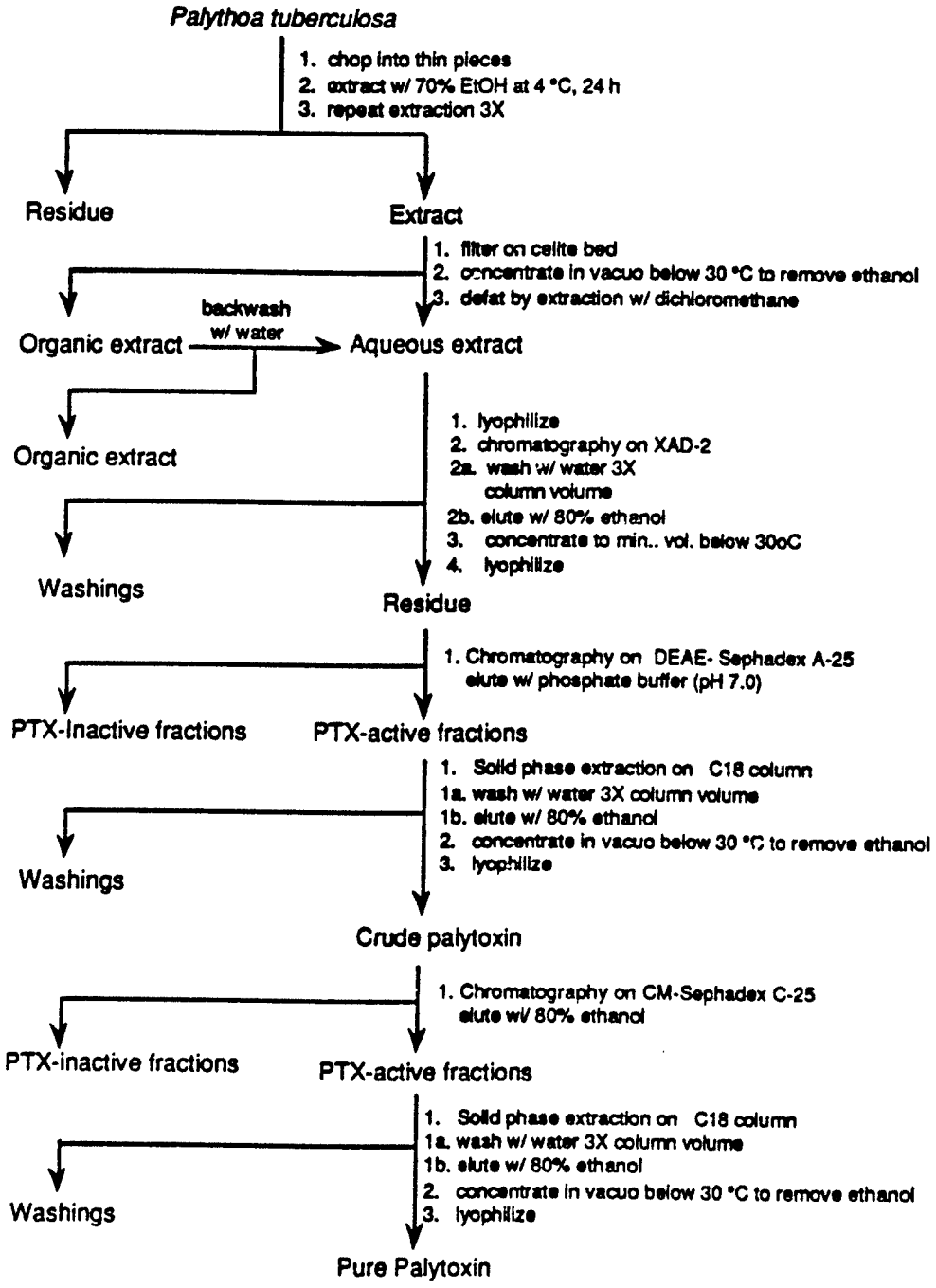
C.8. Wash with 0.2M NaH_2PO_4, pH 4.5 (4x gel volume).

C.9. Finally, wash gel with 0.02M NaH_2PO_4, pH 4.5 (4x gel volume).

C.10. Store the CM Sephadex gel in 0.02M NaH_2PO_4, pH 4.5 with 0.04% azide at 4 °C until needed.

II. ISOLATION PROCEDURE

A. ISOLATION SCHEME



B. EXTRACTION

B.1. Prepare the work area. Clear away all other equipment under the hood. **Absolutely no food or drink is to be within the vicinity.**

B.2. Bags of the frozen coral are allowed to thaw for 1 hour in the sink prior to chopping. An entry must be made in the *Palythoa* logbook for each bag withdrawn.

B.3. Position the large carboy on a cart next to the hood. Make certain that the inlet end of the C-flex (chemically resistant) peristaltic pump tubing is in place on the bottom of the large carboy. (See Figure 1).

B.4. With the blender, chop the sample into small thin pieces, covering the coral with 70% EtOH and running the blender at low speed for about 30 seconds each time. For the succeeding runs, use the same liquid from the first run to cover the coral to minimize final liquid volume.

B.5. After all the coral has been chopped and transferred to the carboy, add enough 70% EtOH to completely cover the coral. Make sure that the outlet of the pump tubing is below the level of the liquid (Figure 1). This is necessary to avoid air oxidation of the palytoxin.

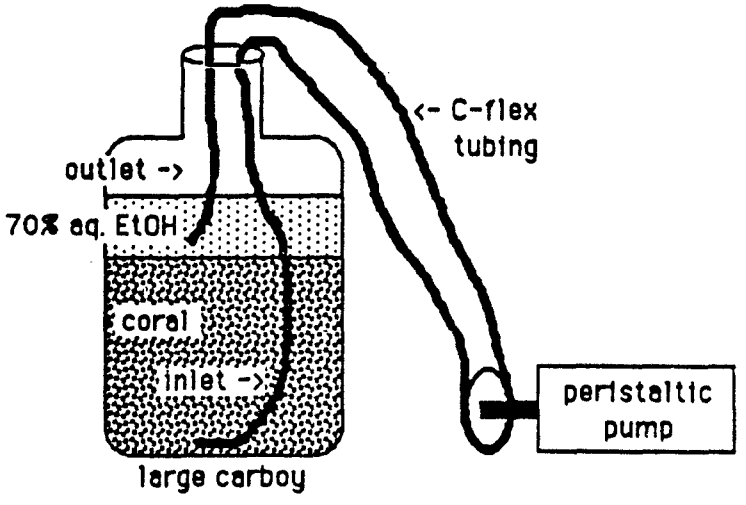


Figure 1. Extraction set-up.

B.6. Using a cart, tranfer the carboy to the cold room and connect the tubing to the pump.

B.7. Place a safety shield in front of the apparatus and start the peristaltic pump.

B.8. Thoroughly clean hood area and all contaminated equipment with 10% aqueous bleach. Properly dispose of all trash, including gloves and paper towels, following decontamination with Clorox.

B.9. After 24 hours transfer the extract, via the pump, to a large flask (Figure 2). Filter through a bed of Celite on a Büchner funnel. The filter tends to clog, change it when necessary.

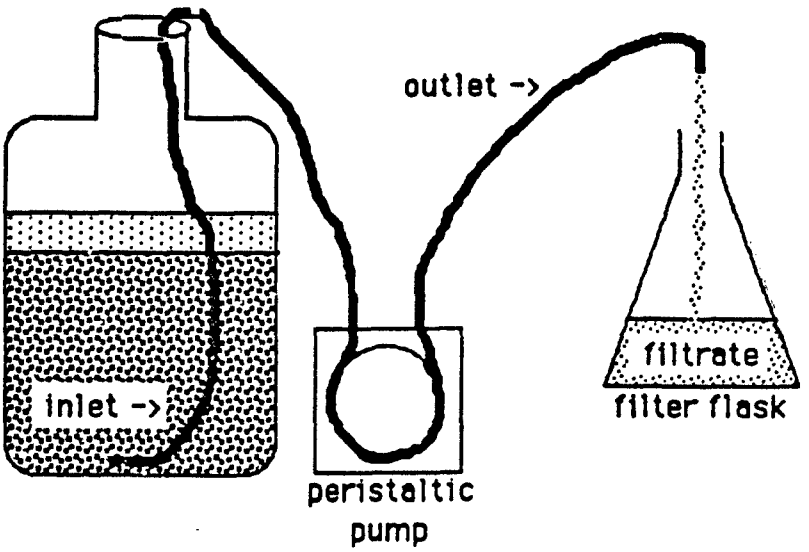


Figure 2. Transfer of extract from carboy.

B.10. Concentrate the filtrate on the R-151 rotovap. The filtrate should be transferred to the evaporating flask via the teflon feed tube. This precaution obviates the need to disconnect and handle the heavy 10 L evaporating flask, thus minimizing the chance for inadvertant exposure.

Note: The liquid tends to bump violently. Watch closely and use great care in adjusting vacuum.

B.11. Repeat extraction/filtration/concentration 3X, adding each successive extract to the first.

B.12. Concentrate the combined filtrate down to a maximum volume of about 800 ml.

B.13. Before removing the evaporating flask, carefully rinse the rotovap steam duct with water to wash all traces of extract back into the flask.

B.14. Store the concentrated extract in the cold room until the next step can be started.

C. DEFATTING:

C.1. Reserve a 1 ml sample of the crude aqueous extract for CIEIA testing. Transfer the remaining extract into a 4L separatory funnel. Rinse the flask several times with deionized water and with CH_2Cl_2. Add rinses to funnel

NOTE: Do not use EtOH to rinse the flask. EtOH tends to stabilize emulsions which prevent clean separation of phases during the extraction.

C.2. Add CH_2Cl_2, (about 1/3x volume of the aqueous extract) to the separatory funnel and gently shake. (**NOTE**: There can be a considerable pressure build-up, release pressure frequently) Let mixture stand in the funnel until there is a clear separation between the two layers (it may take 1-2 hours for the emulsion to dissipate).

C.3. Drain the CH_2Cl_2 layer into another flask. Drain any remaining emulsion together with the organic layer.

C.4. Repeat CH_2Cl_2 extraction 4-5X or until the organic layer is almost colorless.

C.5. Back-extract the combined CH_2Cl_2 extracts with deionized H_2O (3 X 100 ml) to maximize recovery of palytoxin. Add all the washings to the aqueous layer, including the emulsion layer from the final back-extract. Save the CH_2Cl_2 layer for testing by CIEIA.

C.6. Concentrate the aqueous layer with the R-151 rotovap to get rid of any trace of CH_2Cl_2 (CH_2Cl_2 destroys the XAD bed). Reduce volume to about 250 ml.

C.7. Transfer remaining solution to lyophilizing flask(s) (do not fill to more than 1/3 of capacity). Using a dry ice-acetone bath, freeze liquid into a thin shell on inside of flask(s). Lyophilize to dryness.

D. XAD COLUMN:

This step separates the lipophilic organic materials, including palytoxin, from the inorganic salts and non-lipophilic biomolecules.

D.1. Plug the bottom of the column with glass wool. Fill the column half-way with deionized water, open the stopcock and slowly pour the slurry of XAD resin down the side of the column (Figure 3). Make sure there are no air bubbles trapped in the bed. After the column is packed to the desired volume or height, continue running the column with deionized water.

D.2. Dissolve the dried extract with a minimal volume of deionized water.

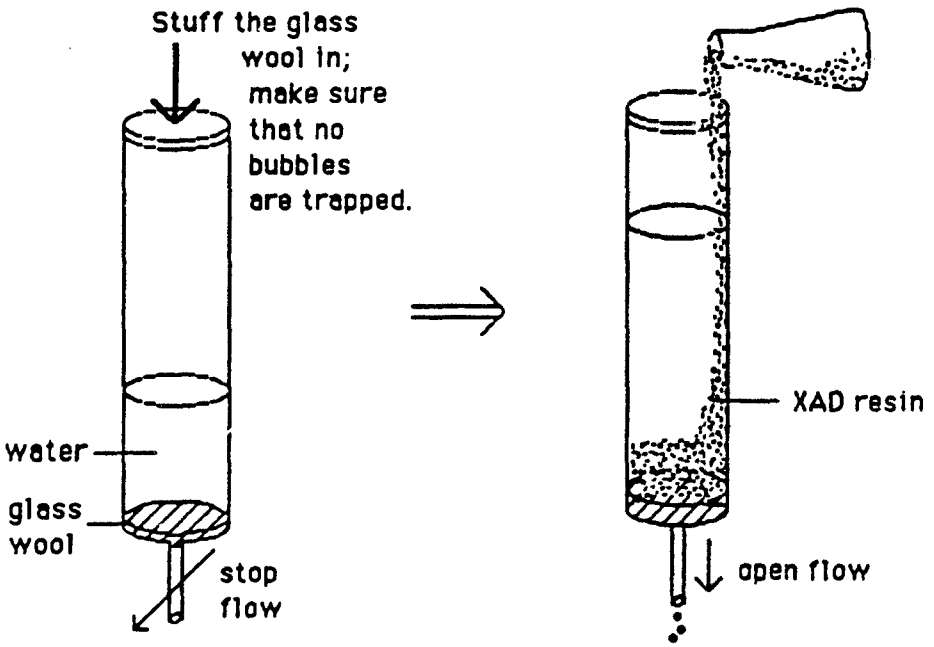


Figure 3: Packing XAD column

D.3. Run the water down to the top of the XAD bed. With reduced flow rate, apply the extract immediately, washing all residue from the flask as quickly as possible (be careful not to run the column dry).

D.4 Continue adding water until one column volume of the effluent is collected. Re-apply this aqueous effluent 6X.

D.5. Wash the column with deionized water (6X column volume). The aqueous flow-through and washings should be combined and set aside.

D.6. Elute the palytoxin from the XAD column with 80% aqueous EtOH. Collect the EtOH extract. Continue eluting with aqueous EtOH until effluent is pale yellow.

D.7. Concentrate the eluant to about 150ml. Reserve ~200 µl for analysis by CIEIA. Lyophilize to dryness.

D.8. Concentrate the combined aqueous flow-through and washings to ~800 ml. Test for palytoxin by CIEIA. If negative, discard. If positive, lyophilize and combine with crude extract of next isolation.

III. PURIFICATION OF CRUDE TOXIN

A. DEAE SEPHADEX A-25 COLUMN:

This is an anion exchange chromatographic purification. The resin contains positively charged groups at this pH. Therefore, negatively charged molecules (acids) are retained and positively charged molecules (bases) are not retained. Since palytoxin possesses a basic amino group and no acidic functions, it eluts in the early fractions.

A.1. Let the DEAE gel warm to room temperature before packing the column.

A.2. Prepare fresh 0.02 M phosphate buffer (pH 7) just prior to packing the DEAE column. Plug the bottom of the column with glass wool. Make sure that no air bubbles are trapped in the column. **HINT**: Fill half of the glass column with buffer and let it flow while pouring in the slurry of DEAE gel down the walls of the column (Figure 4).

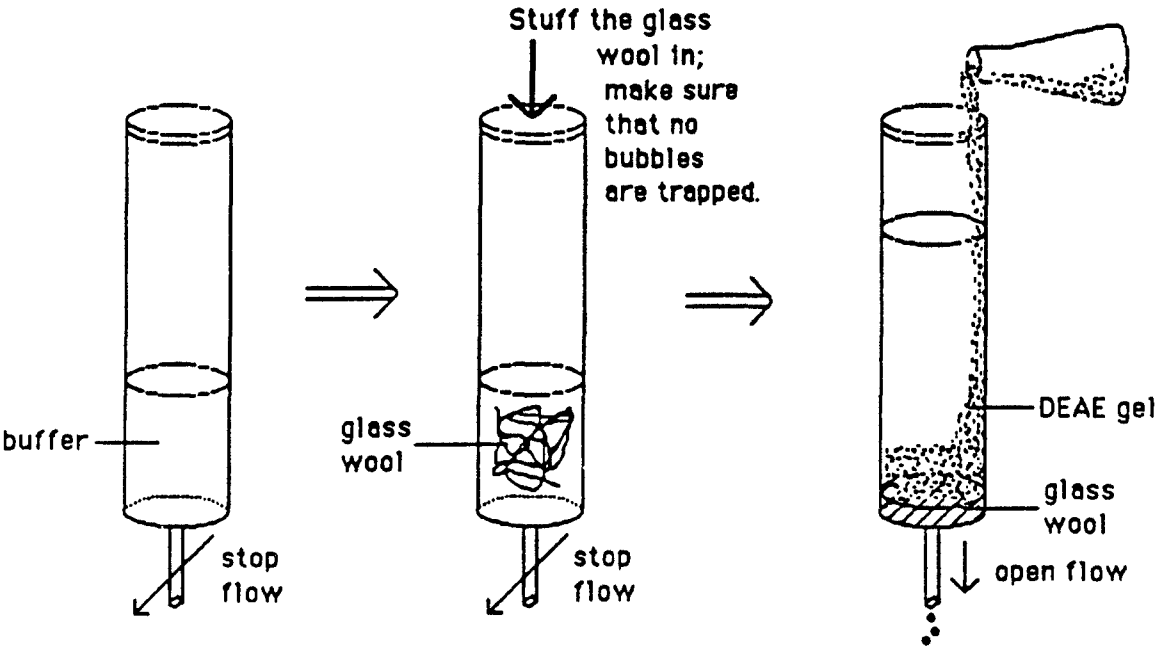


Figure 4: Packing DEAE column

A.3. After the column is packed to the desired height, set up the column as in Figure 5. Ensure that buffer volume in the reservoir is sufficient and let run overnight to equilibrate the column.

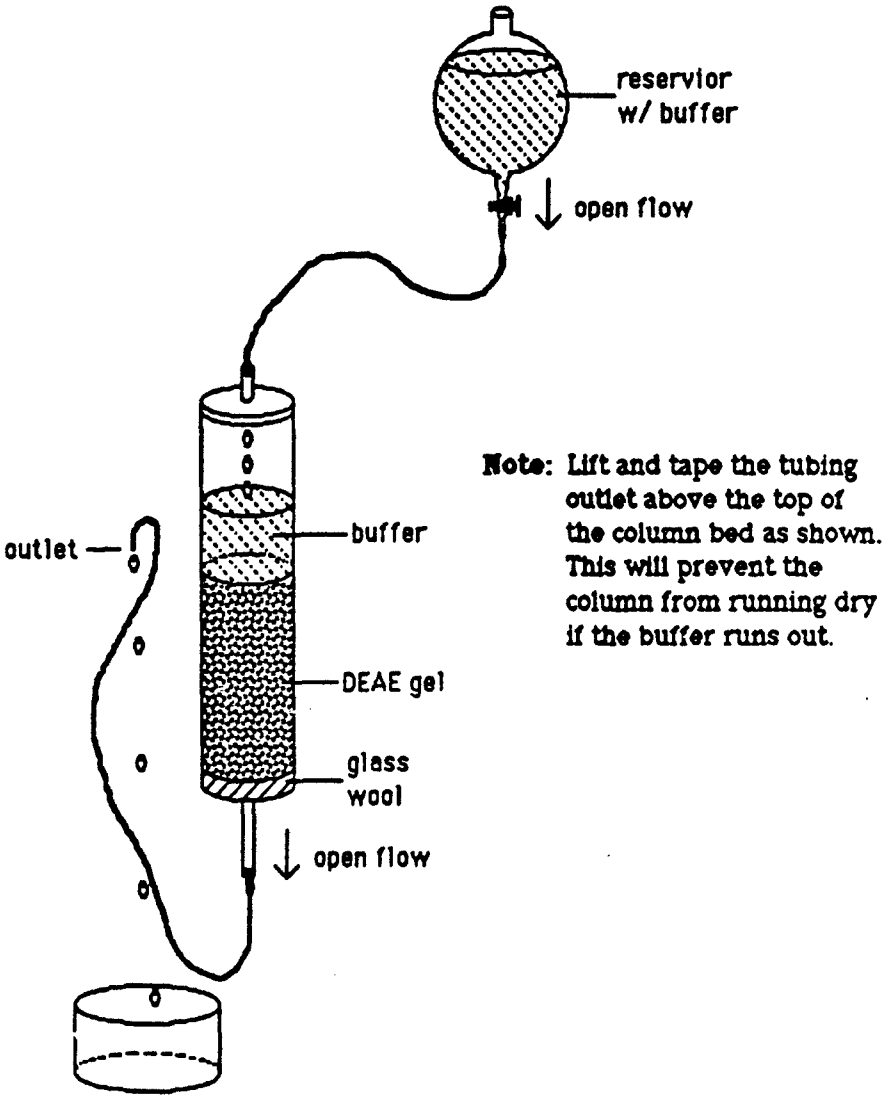


Figure 5: Equilibrating DEAE column.

A.4. While the column is equilibrating, calculate the approximate fraction number at which the palytoxin is expected to come off based on Moore's published information on a given DEAE column size and fraction volume collected.

a) Measure the dimension of the DEAE bed and calculate the volume ($V = hpr^2$).

b) Set the fraction collector at 250 drops/tube (8ml), and the UV detector at 1.0 AUFS, wavelength at 254 nm.

c) Using Moore's published information: (for a DEAE column of 33 cm high X 2 cm diameter, the volume = 103.7 cc. PTX was in fractions #8 - 10; 5 ml/ fraction), calculate the theoretical fraction number at which palytoxin would be expected to come off based on the actual column size and fraction size collected:

$$\frac{\text{volume before PTX eluts}}{\text{bed volume}} = \text{constant}$$

$$\therefore \frac{(5\text{ml/fraction})\ (8\text{ fractions})}{103.7\text{ ml}} = \frac{(8\text{ ml/fraction})\ (\chi\text{ fractions})}{\text{column volume}}$$

∴ Palytoxin is expected to come out in the χ^{th} fraction of 8 ml.

A.5. Column equilibration is complete when the effluent pH ≤ 7.57. Connect the column to the UV detector and continue running the buffer to establish a flat baseline.

A.6. Dissolve the dried extract with a minimal volume (≤ 5% of column volume) of the buffer.

A.7. Run the buffer down to the top of the DEAE bed and with a pipet, apply the extract carefully along the sides of the column. Quickly rinse all extract from the flask onto the column with a minimum volume of buffer so as to minimize tailing. As soon as the sample is applied, start collecting fractions.

A.8. Let all the extract run down to the top of the DEAE bed then rinse the sides of the column and start running the buffer into the column.

A.9. Based on the calculated fraction number for elution of palytoxin (if you are using the UV detector, watch for the peak), confirm which fractions contain palytoxin by obtaining a UV spectrum.

a) Set the UV/Vis spectrophotometer to SCAN mode and the range to 190–300 nm.

b) Using an "Eppendorf" pipetter, withdraw 5-10 µl from a fraction. Add this to water in the UV cuvette and obtain the UV spectrum.

c) Take a UV spectrum of each fraction beginning several fractions before and continuing for several fractions after those thought to containing palytoxin.

d) The UV spectrum of acceptable fractions should look like Figure 6:

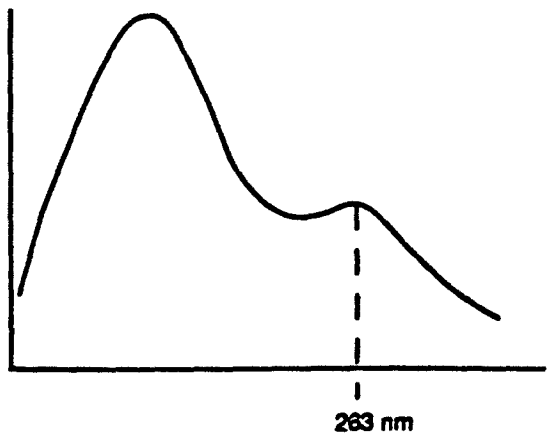


Figure 6. UV spectrum of good palytoxin fractions from DEAE column.

Note the characteristic peaks at 263 and 233 nm, however, as impure as it is at this stage, the UV spectrum of the palytoxin containing fractions may not show the peak at 233 (Figure 7). These fractions are also acceptable.

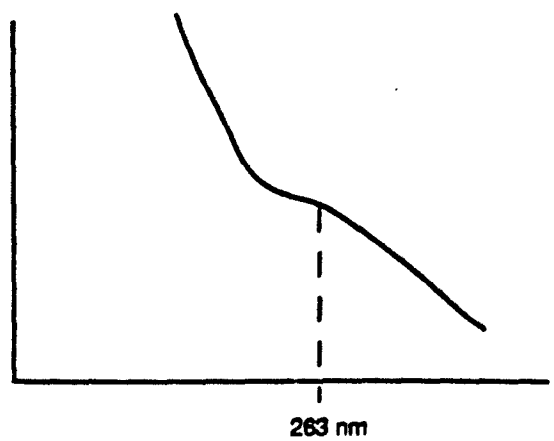


Figure 6. UV spectrum of typical palytoxin fractions from DEAE column.

A.11. Combine all these palytoxin containing fractions and immediately desalt on a pre-packed C-18 SPE column.

B. DESALTING BY SOLID PHASE EXTRACTION

This solid phase extraction is a small scale version of the XAD desalting procedure. A pre-packed C-18 column (Mega Bond Elut by Analytichem International) is used for desalting the DEAE fractions. Organic compounds are strongly retained by the hydrophobic adsorbent in water, whereas the inorganic buffer salts are not retained. The organic compounds are then eluted with aqueous ethanol.

B.1. Set up the column with a vacuum pump as shown in Figure 8.

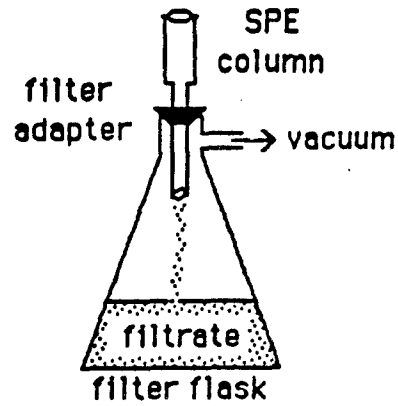


Figure 8: SPE column setup.

B.2. Wash a 60 ml C18 Mega Bond Elut column with methanol (5 X column volume) followed by deionized water to rinse off all traces of methanol.

B.3. Apply the combined DEAE fractions onto the column. Re-apply the collected effluent several times to ensure all the palytoxin had been adsorbed by the column.

B.4. Wash the column with deionized water 3X volume.

B.5. Elute the palytoxin with aqueous 80% EtOH. Palytoxin is strongly retained on C-18. For this size of column, 1.5 liters or more of 80% aqueous EtOH is required to ensure complete elution of toxin from the adsorbent.

B.6. Collect the EtOH extract in a pear-shaped flask and concentrate on the rotovap to remove the ethanol.

B.7. Reserve ~200 µl for analysis by CIEIA. Freeze the remaining solution and lyophilize to dryness.

C CM-SEPHADEX C-25 COLUMN:

This is a cation exchange chromatographic purification. The resin contains negatively charged groups at this pH. Therefore, positively charged molecules (bases) are retained and negatively charged molecules (acids) are not retained. Since palytoxin possesses a basic amino group it is retained on the column and elutes in a later fraction.

NOTE: CM-Sephadex gel should be stored in 0.02 M NaH_2PO_4 (pH 4.5) with 0.02% NaN_3 in the refrigerator.

C.1. Bring the CM-Sephadex gel to room temperature.

C.2. Pack the CM column in the same manner as the DEAE column (Figure 4), but use freshly prepared 0.02 M NaH_2PO_4 (pH 4.5).

C.3. Let the column equilibrate until the effluent pH < 5.7 (it may take up to 4 days to equilibrate the column). Use the same set up as for the DEAE column (Figure 5).

C.4. While the column is equilibrating, calculate the fraction numbers to be collected. This is dependant upon the size of the CM bed and the fraction size.

a) Measure the dimension of the CM bed and calculate the volume ($V = hpr^2$).

b) Set the UV detector at 254 nm and 0.5 AUFS and the fraction colector at 150 drops per fraction (about 4 ml).

c) Using Moore's published information: the column volume = 105.2 cc. PTX was in fractions #18 - 28; 10 ml/fraction.

$$\frac{(10 \text{ ml/fraction})\ (18 \text{ fractions})}{105.2 \text{ ml}} = \frac{(4 \text{ ml/fraction})\ (\chi \text{ fractions})}{\text{column volume}}$$

$\therefore$ Palytoxin is expected to come out in the χ^{th} fraction of 4 ml.

d) Based on the actual column size in use and the fraction size to be collected, calculate the theoretical fraction number at which the palytoxin will come off the column.

C.5. As soon as the column is ready (correct effluent pH and flat baseline on detector) dissolve the dried extract with the very minimal volume (5% of column vol.) of NaH_2PO_4.

C.6. Run the solvent to the top of the column bed and with a pipet, apply the sample into the column as quickly as you can (careful not to run the column dry), wash off the flask and apply immediately into the column to reduce tailing effect. Rinse the sides of the column and continue running the column with the NaH_2PO_4 solution.

C.7. As soon as the sample is applied, start collecting fractions.

C.8. Obtain a UV spectrum of all fractions corresponding to peaks observed with the UV detector (254 nm). Check a few fractions before and after the peak. UV spectra of pure (≥99%), acceptable quality (≥95%), and unacceptable palytoxin samples look like Figures 9-11.

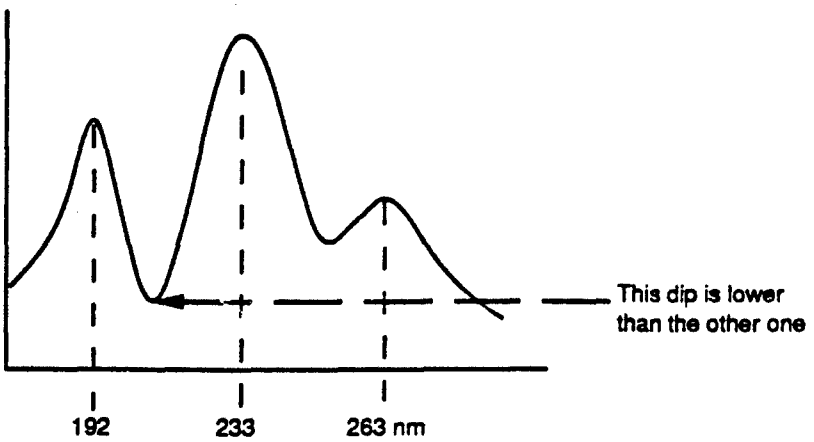


Figure 9. UV Spectrum of ≥99% Pure Palytoxin

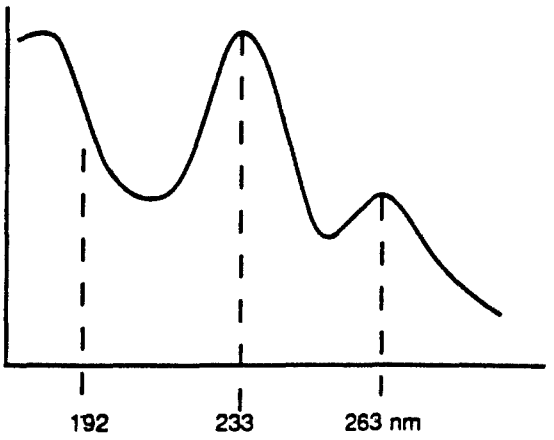


Figure 10. UV Spectrum of ≥95% Pure Palytoxin

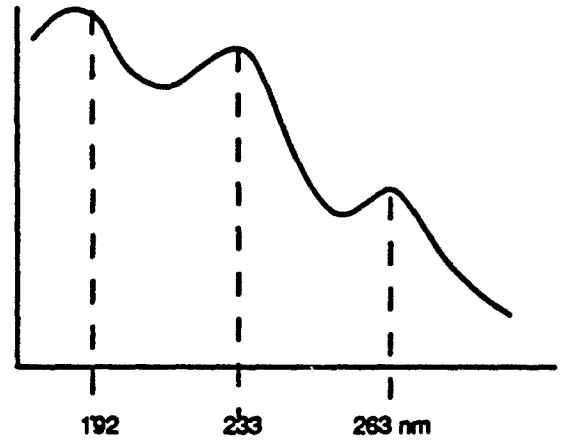


Figure 11. UV Spectrum of Unacceptable Palytoxin

C9. Combine the fractions accordingly into three separate flasks and desalt each of them on a 20 cc size prepacked C-18 SPE column.

D. DESALTING BY SOLID PHASE EXTRACTION

D.1. Prepare the same set-up as in the desalting of the DEAE fractions using the 20 cc pre-packed C-18 Mega Bond Elut by Analytichem International.

D.2. Follow steps B.2 thru B.7 on each of the three samples.

D.3. The impure sample can be further purified by repeating the CM column column and desalting.or saved and added to the next isolation.

IV. CHARACTERIZATION:

A. QUANTITATION BY UV.

A.1. Dissolve the dried extract with 20 ml. of deionized water.

A.2. With a positive displacement pipet, withdraw 20 microliters sample and transfer into a cuvette.

A.3. Add 1.0 ml. of deionized water (measured with a gas-tight syringe)into the cuvette, cover with parafilm and mix the palytoxin sample gently for uniformity.

A.4. Scan sample on the UV spectrophotometer at 263 nm.

A.5. Calculate the palytoxin isolated by using the following data and Beer's Law:

$$A = abc$$

where: A = absorption

a = molar absoprtivity of PTX at 263 nm = 23,600

b = path length = 1.0

c = concentration in M

MW of PTX = 2678.5 g/mole

$$\therefore c(mg) = \frac{A}{ab} \times MW(PTX) \times 1000$$

$$= A \times 113.49576$$

To calculate PTX from total sample solution:

$$c(mg) = A \times 113.49576 \times DF \times V(L)$$

where: DF = dilution factor = $\frac{20\ \mu l + 1000\ \mu l}{20\ \mu l}$

if V (L) = Total volume in liters 0.02

B. QUANTITATION BY CIEIA

All reseved samples should be given to the Immunology group for CIEIA analysis. The result should be within 1-2% of the answer obtained by UV.

C. HPLC ANALYSIS

The purified palytoxin should be checked by HPLC using the following method.

Column:	4.6 X 250 mm Zorbax ODS
Mobile Phase:	1:1 acetonitrile/0.05 N acetic acid
Flow Rate:	1 ml/min
Detector:	UV 263 nm

A representative chromatograph is shown in Figure 13.

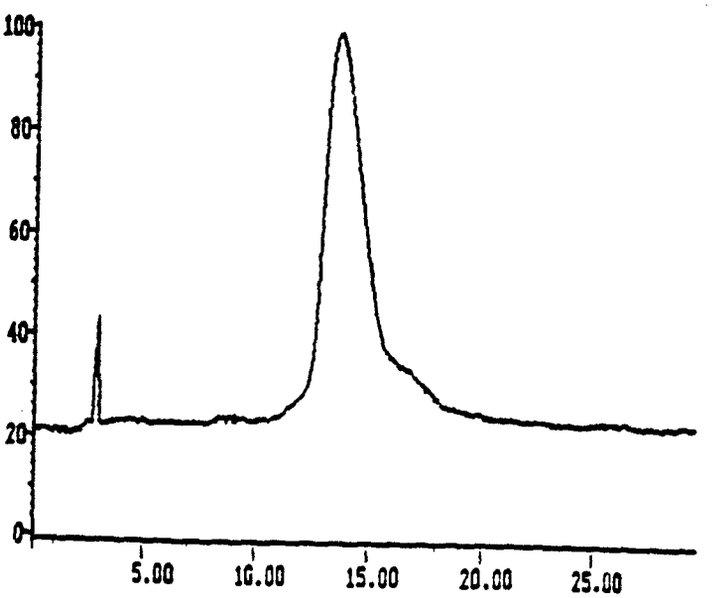


Figure 13. HPLC trace of palytoxin.